REMARKS

i. Status of the claims

Claims 1, 3-6, and 9-25 are pending. Claims 2, 7, and 8 are canceled without prejudice or disclaimer. Applicants reserve the right to file one or more continuing applications directed to canceled subject matter. Claims 6 and 10-24 are withdrawn. Claims 1, 3-5, 9 and 25 are amended for the reasons elaborated below.

Applicants take this opportunity to thank Examiner Ton and Examiner Woitach for courtesies extended during a telephonic interview on May 3, 2005, when the present claims and the cited prior art were discussed. The present amendments reflect the substance of that discussion.

Claims 1 and 5

Claims 1 and 5 are revised to delete "trans-chromosomic nonhuman mammal." Instead, the claims now are directed to a "mouse," which is supported in the specification, *e.g.*, at page 8, lines 14-18, and at page 10, lines 7-9.

Claims 1 and 5 also are amended to specify that the recited human chromosome fragment is "not integrated into the mouse cell genome." Explicit support for this amendment appears, for instance, at page 16, lines 18-22 and in Figures 12 and 16.

Finally, claims 1 and 5 are amended to qualify the "P450 gene" as a member of the "P450 3A family." Support for this recitation is found in original claim 2 (now canceled) and in the specification, for example, at page 18, lines 12-19, and in Figure 3.

Claims 2, 4, and 9

Claims 2, 4, and 9 are amended to ensure correct antecedent basis with the preceding claims. Thus, the amended claims recite "mouse" instead of "trans-chromosomic nonhuman mammal."

Claim 25

Claim 25 is revised to clarify that expression of the "P450 3A family" gene is regulated by an "expression control region" present in the particular P450 3A gene. Support for this amendment can be found at page 14, line 24, of the specification.

Since all of these amendments are fully supported by the application and do not introduce new matter, Applicants respectfully request their entry into the present claim set.

ii. The rejection of claims 1-5, 8, 9 and 25 under Section 112, first paragraph is moot, since the claims no longer recite "trans-chromosomic non-human mammal"

Claims 1-5, 8, 9 and 25 under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the written description requirement is moot, since the claims no longer recite "trans-chromosomic non-human mammal." Accordingly, Applicants respectfully request that this rejection be withdrawn.

iii. Claim 25 does not constitute new matter but has been amended to clarify that the P450 3A gene is regulated via its own expression control region

Claim 25 is rejected under 35 U.S.C. § 112, first paragraph as allegedly introducing new matter. According to the Examiner, the claim "encompasses both promoters endogenous to the human cytochrome P450 gene and other promoters that would [also] induce the expression of the gene." Office Action at page 4.

Applicants have amended claim 25 to clarify that the P450 3A gene is regulated via its own "expression control region." As Applicants indicated above, this amendment is supported at page 14, line 24 of the specification.

It is clear, therefore, that the compound recited in claim 25 regulates the recited P450 3A gene via that gene's own expression control region. Accordingly, Applicants assert that no new subject matter has been introduced into claim 25, and they request withdrawal of this rejection.

iv. The present claims require that the human chromosome fragment "is not integrated into the mouse cell genome" and, therefore, the Office's prior arguments are not considered germane

The Examiner has maintained a rejection of claims 1-5 and 9 under the first paragraph of 35 U.S.C. § 112, for alleged failure to comply with the written-description requirement. According to the Examiner the claims as previously amended "broadly encompass both animals which are transgenic ... and animals which have an extra chromosome." There is no limitation in the claims, the Examiner says, which recites that the human chromosome fragment "is or is not part of the genome of the mammal." Office Action at page 7. "As such, the cited art of record and prior arguments presented in the previous Office action are considered germane" (emphasis added). Office Action at page 7.

Applicants have amended claims 1 and 5 to make evident that the human chromosome fragment in the mouse cell "is not integrated into the mouse cell genome." Literal support for this phrase is found at page 16, lines 18-22. Accordingly, the claims recite an explicit distinction between the claimed mouse and the art of record, obviating previous Office arguments.

v. The present claims are enabled for CYP3A genes; determination of a threshold level of P450 gene expression is unnecessary

Claims 1-5, 8, 9, and 25 stand rejected for alleged non-enablement under 35 U.S.C. § 112, first paragraph. The Examiner concurs, however, that the specification is "enabling for a trans-chromosomic mouse whose genome comprises a human CYP3A4 gene." Office Action at page 8.

(a) *The present claims are directed to CYP3A genes specifically*

Applicants disagree with this interpretation, but, for the sake of expediting prosecution, they have amended the claims to highlight a "P450 3A family" gene. The specification describes several 3A family genes and clusters. See page 18, lines 12-19 and page 43, lines 18-20, for instance. Applicants believe, therefore, that the application describes several P450 3A family species to support the recited element.

(b) *Determination of a threshold level of P450 gene expression is not an essential aspect of the claimed invention; it is only necessary to screen for the presence of the introduced non-host chromosome in a mouse produced by microcell fusion*

The Examiner asserts that the specification "fails to provide sufficient guidance or teachings with regard to how to use the claimed animals. For example, the claims encompass [a] mammal, wherein one cell has the chromosome fragment. There is no teaching ... with regard to how many cells which would have to have the fragment in order to practice the claimed invention." Office Action at page 12.

Furthermore, the Examiner says, there is no teaching "to show how many cells, and how much expression from those cells would be sufficient to produce the effect of the expression of the CYP3A4 enzyme upon administration of the enzyme's substrate." Office Action at page 13.

Applicants would emphasize, however, that determining threshold levels of gene expression is not a primary concern for those who practice microcell fusion in the context of creating trans-chromosomic animals. That is, most researchers are satisfied that a mouse produced by microcell fusion is suitable for metabolism studies if it simply tests positive for expression of a heterologous P450 gene. Hence, a threshold level of P450 gene expression is not an essential aspect of the claimed invention.

(i) Yoshida *et al.*, *J. Nat'l Cancer Inst.* 92: 1717-30 (2000)

The appended review by Yoshida *et al.* (2000) describes microcell-mediated chromosome transfer, for investigating metastasis-suppressor proteins and genes. The Yoshida disclosures comport with the understanding, previously mentioned, that a threshold level of P450 gene expression is not an essential aspect of the claimed invention.

Yoshida reports that "the majority of metastasis-suppressor activities identified to date have been discovered using microcell-mediated chromosomal transfer." The choice of the microcell fusion strategy was "logical," Yoshida says, because "the existence of metastasis-suppressor genes was originally implicated by the results of somatic cell fusion studies, the precursor of MMCT" and because microcell fusion yields "stable hybrids."

The legend of Yoshida's Figure 2 (left-hand column at page 1721) describes the use of A9 donor cells, containing a single human chromosome, for preparation of microcells that will be transferred to the recipient cell lines. "Stable microcell hybrids" are simply selected and characterized by molecular and cellular methods. That is, any one of FISH, karyotyping, and PCR amplification methods can be used to screen for the presence of the introduced chromosome. "Ultimately, the presence or absence of a suppressor region is determined by subcutaneous injection of the hybrids into the flanks of immunodeficient mice."

(ii) Tomizuka *et al.*, *Nat. Genet.* 16: 133-43 (1997)

Similarly, Tomizuka *et al.* (1997), an abstract of which is appended, introduced human chromosomes and fragments into mouse embryonic stem cells via microcell-mediated chromosome transfer and produced viable chimeric mice from them. "Transferred chromosomes were stably retained, and human genes, including immunoglobulin (Ig) kappa, heavy, lambda genes, were expressed in proper tissue-specific manner in adult chimeric tissues" (emphasis added).

(iii) Tomizuka *et al.*, *Proc. Nat'l Acad. Sci., USA* 97: 722-27 (2000)

Likewise, Tomizuka *et al.* (2000) (appended) later used the same methodology to produce "double trans-chromosomic mice." In keeping with previous reports, they mention that the resultant chimeric mice and resultant offspring "were examined by PCR and ELISAs." See right-hand column at page 723, under the "Breeding Analysis" subsection.

Accordingly, there is no question that those who practice microcell fusion to create transgenic animals models are satisfied that the technique produces chimeras in which essentially all cells of the host contain the desired genetic material. This is especially true when embryonic stem cells are used, as is the situation in the present case. In this vein, see Example 2 at pages 62-66 of the application, which example describes the fusion of embryonic stem cells (page 63, lines 20-23) and detection of the introduced CYP3A5 and CYP3A7 genes via PCR (pages 65 and 66).

For the reasons set forth in subsections (a) and (b) above, Applicants assert that the present claims are enabled for a mouse that comprises a human CYP3A4 gene and request that this rejection be withdrawn.

vi. The "indefiniteness" rejection is mooted by deletion of "trans-chromosomic"

Claims 1-5, 8, 9, and 25 are rejected under 35 U.S.C. § 112, second paragraph, for reciting "trans-chromosomic," which the Examiner alleges is unclear. Without acquiescing, Applicants have deleted the term, thereby obviating the stated grounds for rejection.

vii. Li cannot anticipate because it says nothing about a mouse cell in which the desired chromosome fragment is not integrated into the mouse genome

Claims 1-5, 8, 9, and 25 are rejected as allegedly anticipated by Li *et al.*, *Archives of Biochem.* ("Li 1") or Li *et al.*, *Biochem. Biophys. Res. Comm.* ("Li 2"). According to the Examiner, the previously amended claims "as broadly written, encompass the mice as taught by Li, because Li's mice have cells that have a human chromosome fragment." Office Action at page 14.

The Examiner also stated that Applicants argument, that Li does not teach a fragment "outside of the host's cellular genome," is inapposite because Applicants "are arguing limitations that are not found in the claims." Office Action at page 15. Thus, the Examiner urged that "there is no recitation of whether the chromosome fragment exists within the mammal's genome or not."

Both Li documents teach the direct integration of the CYP3A7 gene into the host cell genome. Neither document says anything about introducing the CYP3A7 gene into the host cell such that the gene remains distinct from (not integrated into) the host genome. Indeed, such a concept would be counterintuitive, since the whole point of Li is to create stable, transgenic mouse cell lines that contain the CYP3A7 gene.

Thus, Li 1 states "we established several transgenic mouse lines carrying a CYP3A7 cDNA driven by a murine MT-1 promoter" (page 312, lines 14-15 of the first paragraph). Likewise, Li 2 results demonstrated that "the CYP3A7 gene has been integrated into the mouse genome." See page 235, left-hand column.

Accordingly, neither Li 1 nor Li 2 teaches "a human chromosome fragment that is not integrated into the mouse cell genome," as presently recited. Li 1 and L 2 do not anticipate the present claims, therefore, and Applicants request withdrawal of this rejection.

viii. Conclusion

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested. The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Respectfully submitted,

Date 25 May 2006

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REVIEW

Metastasis-Suppressor Genes: a Review and Perspective on an Emerging Field

Barbara A. Yoshida, Mitchell M. Sokoloff, Danny R. Welch, Carrie W. Rinker-Schaeffer

Metastasis is the most lethal attribute of a cancer. There is a critical need for markers that will distinguish accurately those histologic lesions and disseminated cells with a high probability of causing clinically important metastatic disease from those that will remain indolent. While the development of new diagnostic markers of metastasis was the initial motivation for many studies, the biologic approach used to identify metastasis-suppressor genes has provided surprising insights into the *in vivo* mechanisms regulating the formation of metastases. This review and perspective describes the evolving view of the mechanisms that regulate metastasis and the importance of metastasis-suppressor genes in this process. The known metastasis-suppressor proteins or genes and the microcell-mediated chromosomal transfer strategy used to identify many of them are reviewed. New evidence for the role of these metastasis-suppressor proteins or genes in regulating the growth of disseminated cancer cells at the secondary site, the potential for the identification of novel therapeutic targets, and the multidisciplinary approach needed to translate this information into clinical tools for the treatment of metastatic disease are discussed. [J Natl Cancer Inst 2000;92:1717–30]

Clinical Problem: Predicting Metastatic Propensity

Our ability to detect and successfully treat localized cancers has improved appreciably in recent years. However, metastatic disease presents a continuing therapeutic challenge and is the most common cause of cancer-related death. Thus, there is an emphasis on the diagnosis of cancers at an early stage, when they are localized and most likely to be curable. Although screening for early-stage disease is logical, its utility is limited by the inability of conventional diagnostic and histologic parameters to predict accurately the true extent and prognosis of a substantial proportion of clinically localized cancers *(1–3)*. This limitation is due, in part, to the inherent limitations and subjectivity of current grading and staging systems *(4,5)*.

The incidence of disease recurrence in surgical patients treated for prostatic and breast cancers illustrates this problem particularly well. Although we have a wealth of clinical and biologic information on these diseases, a large percentage of apparently resectable and theoretically curable lesions is found to be more advanced at the time of resection than envisaged, resulting in a substantial failure rate after attempted curative surgery *(6–8)*. In studies of prostate cancer patients *(9–11)*, even when patient selection excludes men with factors predicting poor prognosis (e.g., poorly differentiated histology, high prostate-specific antigen [PSA] levels, and clinical suspicion of local invasion), the relapse rate after radical retroperitoneal prostatectomy has approached 20%–30%. Similarly, one third of surgical patients with lymph node-negative breast cancer will develop metastases, while the other two thirds, despite receiving no chemotherapy, will not *(12)*. Even in patients with small tumors and tumor-negative lymph nodes (T1N0), there is a 15%–25% likelihood of distant metastases *(8)*.

Since the current staging systems for breast and prostate cancers do not accurately identify those patients curable by regional treatment alone, the evaluation of additional parameters associated with the metastatic phenotype will be very important for the differentiation of patients curable by surgery alone from those requiring systemic therapy. For instance, men at high risk for relapse of prostate cancer can be identified [e.g., serum PSA level >10 ng/mL; clinical stage T1 or T2 with >50% of tissue at Gleason grade 4 *(3,4)* on biopsy or clinical stage T3 prostate cancer] and would be immediate candidates for adjuvant antimetastatic therapies if they existed *(10,11,13–16)*. Likewise, breast cancer patients with particularly poor prognoses can be identified by the detection of high microvessel counts concurrent with low expression of Nm23 and/or E-cadherin in the primary tumor *(12–17)*. In fact, these parameters are better prognostic biomarkers than the conventional analysis of tumor size and grade. The information obtained from the simultaneous evaluation of biomarkers such as these have the potential to lead to a reduction in the morbidity among those patients not requiring chemotherapy and possibly identify those patients requiring more aggressive therapies than indicated by current methods.

Overall, it is clear that there is a critical need for markers that will distinguish accurately those histologic lesions and disseminated cells that have a high probability of causing clinically important metastatic disease from those that will remain indolent *(5,15)*. Concerns have been raised that "metastasis" has often occurred by the time of diagnosis of the primary tumor, the implication being that it is then too late for antimetastatic therapy to be of use *(18)*. However, the mere spread of cancer cells into the vasculature or to a secondary site does not constitute metastasis. Development of clinically significant metastases requires that a cancer cell complete a series of well-defined steps, generally referred to as the metastatic cascade *(13)*. If a

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See "Notes" following "References."

cell fails to complete any one of these steps, overt metastases will not develop *(13–15)*.

The clinical importance of disseminated cancer cells (detected by sensitive methods such as reverse transcription–polymerase chain reaction [RT–PCR]) has become an issue of considerable interest *(19)*. Several such studies *(16,20)* have reported the detection of tumor-derived cells in the circulation and bone marrow without future development of disease. Other reports have demonstrated an increased risk of disease recurrence in patients with bone marrow micrometastases both for prostate cancer [by the detection of messenger RNA transcripts for PSA *(21)*] and breast cancer [by the detection of cytokeratin-positive cells *(22)*]. Even in these later studies, however, the majority of patients with tumor cell-positive bone marrow samples did not actually develop recurrent disease, although the proportion with recurrence could increase given extended time for patient follow-up. The discrepancy regarding the clinical importance of disseminated cells is likely due to differences in the experimental approaches used to identify cells (i.e., RT–PCR versus immunohistochemical detection).

Tumor cell growth at the site of metastasis is an important clinical target, since cells must survive and proliferate to grow into overt, macroscopic metastases. The first step toward developing effective therapies to inhibit such growth is to identify the genes/proteins that regulate metastatic colonization. To this end, a growing number of laboratories are focusing translational research efforts on the discovery of genes that specifically regulate the metastatic ability of cancer cells. For example, several metastasis-promoting genes—including WDNM-1, WDNM-2, MMP11 (stromelysin-3), MTA1, and ERBB2—have been identified in association with the development of metastatic breast cancer *(23–27)*. One must keep in mind, however, that it takes the coordinated expression of many genes to allow the development of metastases *(28,29)*. Thus, while it is relatively easy to demonstrate an association for a given gene with metastatic ability, it is difficult to prove that a particular gene is essential. On the other hand, it only takes one gene to block metastasis, since inability to complete any step of the metastatic cascade renders a cell nonmetastatic. Metastasis-suppressor genes suppress the formation of spontaneous, macroscopic metastases *without affecting the growth rate of the primary tumor*. It has now been more than 10 years since the discovery of the first metastasis-suppressor gene nm23 (NME1) *(30)*. Since then, both *in vitro* and *in vivo* (e.g., animal) studies *(15,30–32)* have documented the important role of the loss of metastasis-suppressor gene function in the acquisition of metastatic ability.

While the initial motivation for these studies was the development of new diagnostic markers of metastasis, the biologic approach used to identify metastasis-suppressor genes has provided surprising insights into the *in vivo* mechanisms regulating the formation of metastases. We anticipate that identifying the molecular pathways that regulate metastatic colonization and growth control at the secondary site will provide additional, potentially novel therapeutic targets for the treatment of metastatic disease. The purpose of this review is 1) to present the evolving view of the mechanisms that regulate metastasis, 2) to describe the functional strategy used to identify metastasis-suppressor genes and discuss important principles learned from these studies, 3) to document the known metastasis-suppressor genes and report new evidence that supports their role in the regulation of growth control at the secondary site, and 4) to discuss the multidisciplinary approach needed to translate metastasis-suppressor genes into clinical tools.

Regulation of Metastatic Propensity—Evolving Paradigms

Metastasis is defined as the formation of progressively growing secondary tumor foci at sites discontinuous from the primary lesion *(15)*. This process is illustrated by the spontaneous hematogenous metastasis of tumor cells to the lung (Fig. 1, A). The formation of a primary tumor requires a cadre of molecular and

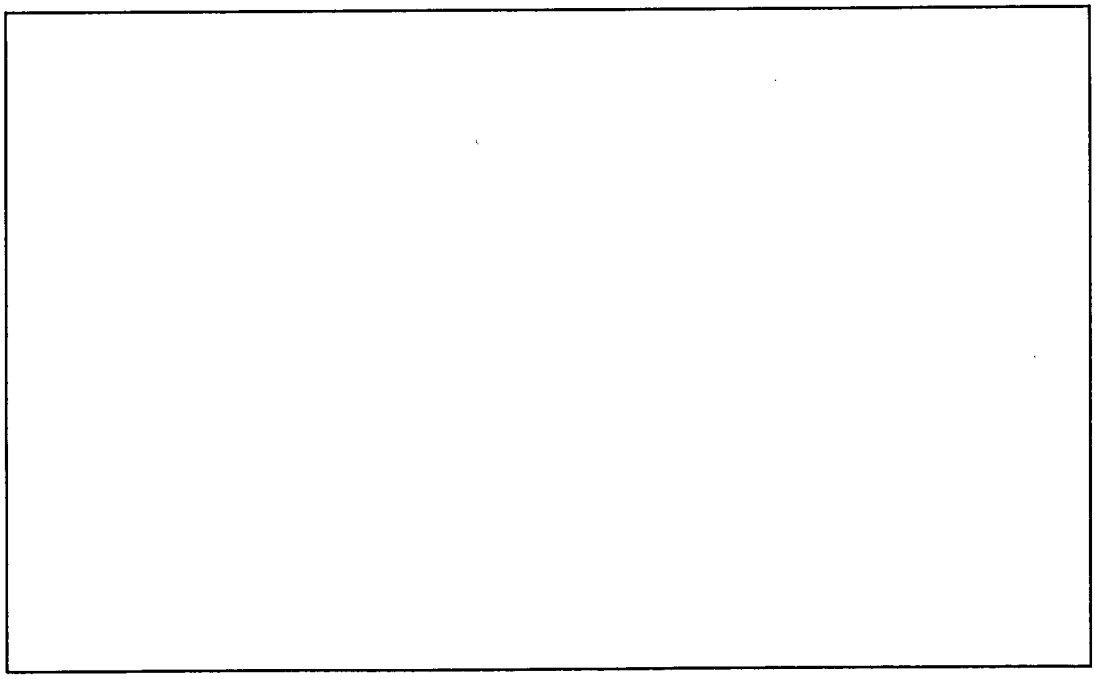

Fig. 1. Development of spontaneous hematogenous metastases. **Panel A:** The development of spontaneous hematogenous metastases requires cancer cells to complete a well-defined series of steps. This figure is adapted from *(13)*. **Panel B:** To form overt metastases, disseminated cells must complete additional steps at the metastatic site(s).

cellular alterations that enable a cell(s) to circumvent normal growth control mechanisms as well as to manipulate its local environment *(14)*. These changes include the development of a blood supply once the focus of transformed cells grows beyond a size that can be nourished by nutrient or metabolite diffusion *(33,34)*. Tumor progression and the acquisition of metastatic competence require additional changes in gene expression (e.g., protein-degrading enzymes and adhesion molecules) that culminate in a malignant phenotype. After invasion into adjacent tissues, tumor cells disseminate via blood vasculature or lymphatics and travel individually or as emboli made up of tumor cells or tumor and host cells. At the secondary site, cells or emboli arrest either because of their physical size or by binding to specific molecules in particular organs or tissues *(15,35)*. For disseminated cells to grow into overt metastases, they must survive and proliferate in the vasculature or in the surrounding tissue after extravasation. The formation of clinically important metastases depends on the completion of *every step of this cascade*, the last of which is metastatic colonization (Fig. 1) *(14)*.

The presence of isolated cells at a secondary site represents a risk to the patient. Cells getting to the secondary site certainly have the potential to colonize; therefore, it is crucial not to ignore the presence of neoplastic cells anywhere. On the other hand, as we will show, the mere presence of cells does not necessarily mean that metastatic colonization will occur. The challenge is to determine how to discriminate between disseminated cells that will form overt metastases from those that will not.

Cancer metastasis, both clinically and experimentally, is known to be inefficient *(36)*. In experimental models, fewer than 0.1% of cells injected into the circulation go on to form secondary tumors *(15,37)*. While many factors contribute to the observed inefficiency of metastasis formation, those considered to be most important include the low survival rates of cells in the circulation and the low percentage of cells that successfully escape from the vasculature into surrounding tissues *(18)*. At this time, there is some question as to whether postextravasational growth control or growth within a vessel are more predominant *(38)*. This process has, for the most part, been studied using assays in which the number and kind of cells injected are known and the numbers and sizes of metastases formed are assessed *(18,39)*. The processes that are responsible for metastatic efficiency *in vivo* remain hidden; thus, mechanistic paradigms have largely been based on logical inference rather than on direct observation. The development of new technologies has enabled researchers to test the possibility that cancer cell dissemination, arrest (nonspecific arrest and/or specific adhesion events), and growth at the secondary site are critical determinants in metastasis formation.

The ability to observe single cells *in vivo* has been greatly enhanced by improvements in intravital microscopy and the use of vital fluorescent dyes like green fluorescent protein (GFP) *(18,40)*. Studies that couple these two powerful techniques have added greatly to our knowledge of the metastatic processes following tumor cell entry into circulatory compartments. The use of *in vivo* video microscopy allows for the direct observation of experimental metastasis over time *(39)*. Cancer cells can be fluorescently labeled *in vitro* and then injected into an animal. The cells can then be viewed at different time points, by both fluorescence and oblique transillumination, in thin tissues or superficial ($\leq$50 μm) regions of thick tissues *in vivo* *(39)*. Experiments using this technology have demonstrated that, in contrast to the long-held belief, the vast majority of cancer cells in the microcirculation manage not only to survive there but also to extravasate into the surrounding tissue within 1–2 days *(41,42)*. Such studies have translated well into the clinical arena. Specifically, the vast majority of clinical studies using RT–PCR to detect prostate tumor cells in the peripheral circulation and bone marrow found no association between the detection of disseminated cells and treatment failure *(16,20)*. Of interest, in a recent study of breast cancer patients *(22)*, detection of cytokeratin-positive cancer cells in the bone marrow was associated with the development of overt metastases and death. The apparent difference between these two findings may be due to differences in study design (e.g., detection methods and markers used) or in factors that influence the growth of disseminated cancer cells at the metastatic site. Additional studies will be necessary to distinguish between these possibilities. Taken together, the clinical and experimental evidence supports the observation that dissemination from the primary tumor site is a frequent event. Furthermore, these independent and complementary studies strongly suggest that growth control of individual disseminated cells determines the efficiency of metastatic colonization.

Metastatic colonization is the lodging and subsequent growth of disseminated cancer cells to form clinically significant metastases (Fig. 1, B). To proliferate, surviving disseminated tumor cell(s) must be able to initiate cell-appropriate, context-dependent signaling cascades, which enable them to survive, enter the cell cycle, and divide. While disseminated cells are likely to be present in numerous organs, only certain environment(s) appear to allow their survival and subsequent growth *(37,43,44)*. Intercellular interactions with the stroma and with other tumor cells are critical for tumor cell survival and involve the activation of adhesion-dependent survival pathways, such as those described for E-cadherin *(45,46)* and integrin molecules *(47)*. Clusters of proliferating cells grow into lesions consisting of a few hundred that can be detected reliably by histologic methods. Cells within such microscopic lesions can receive oxygen and nutrients by diffusion. Progressive growth of microscopic lesions into overt or macroscopic metastases (>1 mm in diameter) requires that the fraction of proliferating cells exceed the fraction that are quiescent or apoptotic. This transition from microscopic to macroscopic metastasis has often been referred to as the switch to an angiogenic phenotype or the angiogenic switch *(48)*. This terminology implies that microscopic metastases exist in one of two states: Either the lesion is angiogenic (forming new blood vessels), or it is not. However, the progression from a "microscopic lesion" to an overt metastasis is more accurately described in terms of growth control. Indeed, the interchangeable use of "angiogenesis" and "growth" has been a source of confusion. This progression may occur over a period of months or even years and is not necessarily dependent on new blood vessel formation. Vascularization is, in fact, a late step in metastatic colonization *(49)*. Recent studies have shown that, in addition to the induction of classical neovascularization via endothelial cell recruitment, tumor cell masses can develop a blood supply by alternative means, such as the cooption of pre-existing host vessels *(49)* or by the formation of tumor channels, a process referred to as vascular mimicry *(50)*. As we will describe in the following paragraphs, recent data from our laboratories suggest that a subset of metastasis-suppressor genes inhibits early steps in metastatic colonization, prior to the need for development or recruitment of vessels.

Identification of Metastasis-Suppressor Activity: A Functional Approach

Metastasis-suppressor genes suppress the formation of (spontaneous) macroscopic metastases. As their name implies, these genes are distinct from *oncogenes*, which promote cellular transformation, and *tumor-suppressor genes*, which suppress tumor growth. While the first metastasis-suppressor gene, nm23, was identified by a complementary DNA (cDNA) subtraction approach, the majority of metastasis-suppressor activities identified to date have been discovered using microcell-mediated chromosomal transfer (MMCT) (Table 1). The choice of the MMCT strategy was logical, since the existence of metastasis-suppressor genes was originally implicated by the results of somatic cell fusion studies, the precursor of MMCT *(51–54)*. The techniques for the generation of genetically stable somatic cell hybrids were developed in the early studies by Barski et al. [reviewed in *(55)*]. In most instances, fusion between malignant and normal cells results in hybrid cells that are suppressed in their tumorigenic capacity *(56)*. Ichikawa et al. *(57)* were the first researchers to identify specific chromosomal losses associated with the reacquisition of metastatic ability. In their study, fusion of nonmetastatic with highly metastatic Dunning rat prostatic cancer cells resulted in nonmetastatic hybrids. More important, the tumorigenicity (e.g., tumor formation and latency period) and *in vivo* growth rates of the primary tumors of hybrid clones containing a full complement of rat chromosomes were not affected. At the experimental end point, none of the animals bearing hybrid tumors developed distant metastases. However, when the nonmetastatic primary tumors were serially passaged *in vivo*, animals occasionally developed distant metastases. Cytogenetic analysis of these metastatic revertants revealed a consistent loss of a copy of rat chromosome 2. This critical study suggested that the loss of specific chromosomes could increase the metastatic potential of prostate cancer cells without affecting growth rate or tumorigenicity.

The observation of a metastasis-suppression activity being associated with a specific chromosome coincided with the development of MMCT as a technique for the study of genes encoded by individual human chromosomes *(51–54,58–61)*. In this approach, summarized in Fig. 2, well-characterized donor cells, carrying a single human chromosome tagged with a selectable marker or markers (e.g., neomycin phosphotransferase, etc.), are used to transfer the chromosome of interest into recipient cells *(62)*. Briefly, donor cells are sequentially treated with Colcemid, to depolymerize microtubules, and cytochalasin-B, to depolymerize actin bundles. The treated cells are centrifuged, and the resulting pellet contains the microcells *(63)*. Microcells are, in effect, micelles that contain a single chromosome or multiple chromosomes. To enrich for those containing a single chromosomes, the microcells are size fractionated by sequential filtration through polycarbonate membranes of decreasing pore size. Microcells become attached to recipient cells in the presence of phytohemagglutinin and then become fused with the addition of polyethylene glycol. Recipient cells containing human chromosomes are selected in G418-containing media and

Table 1. Chromosomal regions identified by microcell-mediated chromosomal transfer that suppress metastases *in vivo**

Chromosomal location	Tumor type or site (reference Nos.)	Cell lines tested (species of origin)	*In vitro* phenotype†	*In vivo* phenotype
Chromosome 1	Melanoma *(72)*	MelJuSo (human)	ND	↓ Spontaneous mets. ↓ Experimental mets.
6q16.3–q23	Melanoma *(73,74)*	C8161 (human)	↓ Motility	↓ Spontaneous mets. ↓ Experimental mets. Occasional single cells (detected by GFP tagging) which are growth suppressed but viable
		MelJuSo (human)	ND	↓ Spontaneous mets. ↓ Experimental mets.
Chromosome 6	Breast *(75)*	MDA-MB-435 (human)	ND	NE spontaneous mets.
7q21–22 and/or 7q31.2–32	Prostate *(76)*	AT6.3 (rat)	ND	↓ Spontaneous mets. ↓ Experimental mets.
8p21–p12	Prostate *(77,78)*	AT6.2 (rat)	↓ Invasion	↓ Spontaneous mets. NE experimental mets.
10cen–10q23	Prostate *(79)*	AT6.3 (rat)	ND	↓ Spontaneous mets.
11q13.1–13.2	Breast *(75,80)*	MDA-MB-435 (human)	ND	↓ Spontaneous mets.
11pter–q14		R1564 (rat)	ND	NE spontaneous mets.
11p11.2–13	Prostate *(68)*	AT6.1 (rat)	ND	↓ Spontaneous mets.
		AT3.1 (rat)	ND	↓ Spontaneous mets.
12qcen–q13 and/or 12q24–ter	Prostate *(64)*	AT6.1 (rat)	NE motility‡ NE invasion‡	↓ Spontaneous mets. No micrometastases observed at the experimental end point
16q24.2	Prostate *(82)*	AT6.1 (rat)	ND	↓ Spontaneous mets.
17p12–11.2 and/or 17cen–q12	Prostate *(63)*	AT6.1 (rat)	NE motility‡ NE invasion‡	↓ Spontaneous mets. Micrometastases observed at the experimental end point

*ND = not determined, NE = not examined, GFP = green fluorescent protein, and mets. = metastases.

†Motility was measured by micropipet motility assay or by migration toward a chemoattractant in Boyden chambers. Invasion was measured by migration through Matrigel.

‡Rinker-Schaeffer CW: unpublished results.

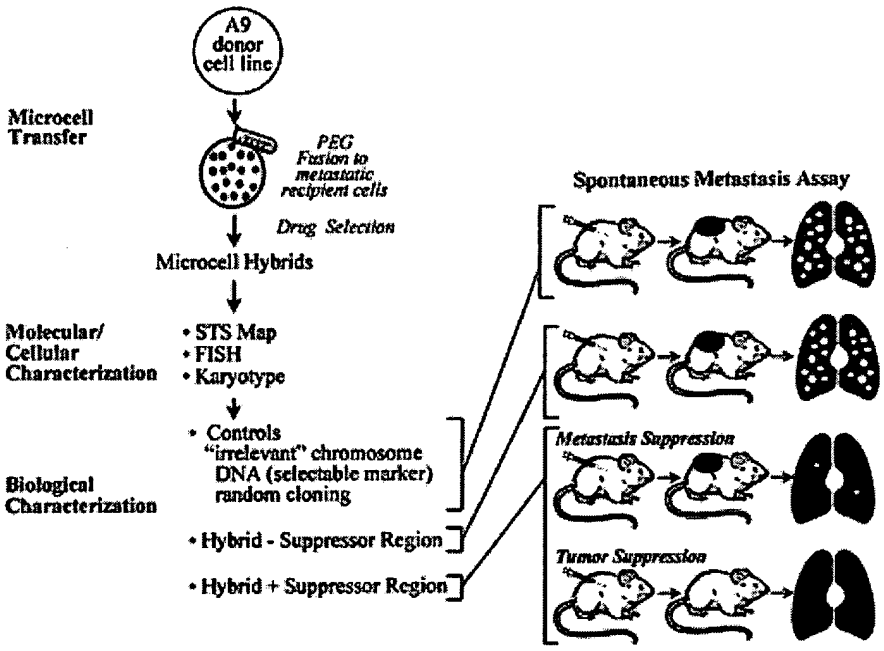
A9 donor cell line
Microcell Transfer
PEG
Fusion to metastatic recipient cells
Drug Selection
Microcell Hybrids
Molecular/ Cellular Characterization
• STS Map
• FISH
• Karyotype
Biological Characterization
• Controls "irrelevant" chromosome DNA (selectable marker) random cloning
• Hybrid - Suppressor Region
• Hybrid + Suppressor Region
Spontaneous Metastasis Assay
Metastasis Suppression
Tumor Suppression

Fig 2.

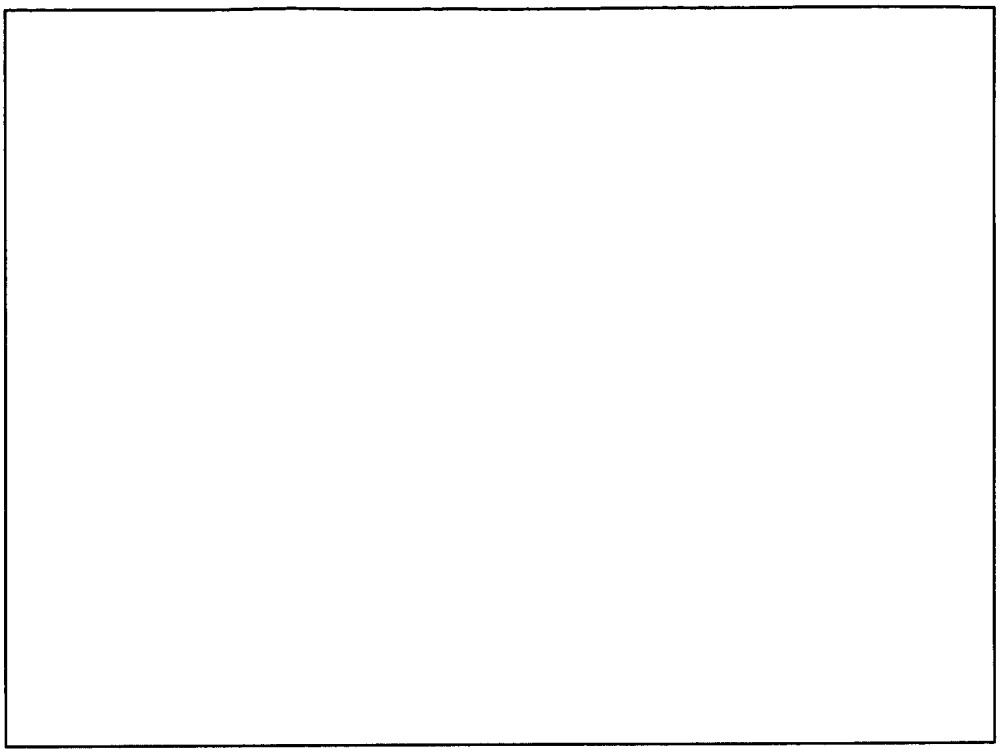

Fig. 2. Identification of metastasis-suppressor activities by use of microcell-mediated chromosomal transfer. A9 donor cells containing a single human chromosome are used for the preparation of microcells that will be transferred to the recipient cell lines. Stable microcell hybrids are selected and characterized by molecular and cellular methods. To screen the microcell hybrids for the minimal metastasis-suppressor region, one employs the following techniques: FISH, karyotyping, and PCR amplification for human specific sequences using STS markers. Ultimately, the presence or absence of a suppressor region is determined by subcutaneous injection of the hybrids into the flanks of immunodeficient mice *(63)*. Inclusion of a variety of controls is critical for the definition of metastasis-suppressor activity *in vivo*. The potential outcomes of *in vivo* studies using control, suppressed, and unsuppressed hybrids are illustrated at **left**. FISH = fluorescence *in situ* hybridization, PCR = polymerase chain reaction, STS = sequence-tagged site, and PEG = polyethylene glycol.

then characterized by molecular and cytogenetic methods, such as sequence tagged-site PCR, karyotyping, and fluorescence *in situ* hybridization *(63,64)*. The complete characterization of the hybrids under study is critical, since it provides information on the addition and/or deletion of donor and recipient chromosomal material, as well as any rearrangements that may have occurred during MMCT.

Several laboratories have employed the technique of MMCT to test the functional significance of chromosomal alterations, such as loss of heterozygosity (LOH), observed in clinical samples. In addition, the use of MMCT, in combination with positional or expression-based cloning techniques, has allowed the functional identification of genes conveying phenotypes, such as senescence or tumor and metastasis suppression *(15,65,66)*. A review of the literature shows that transfer of a given chromosome can have different phenotypic effects that are dependent on the characteristics of the recipient cell line. For example, the transfer of human chromosome 7 by MMCT into immortalized SUSM-1 fibroblast cells induces senescence *(67)*, whereas transfer of the same chromosome into choriocarcinoma cells results in suppression of tumor growth *in vivo (68)*. Such results have enabled the definition of complementation groups for particular chromosome functions. The potential outcomes of transferring a particular chromosome into highly metastatic cells are summarized in Fig. 2.

Studies using highly metastatic Dunning rat prostatic cells as the recipients for chromosomal transfer *(63,64)* showed that chromosomes 12 and 17 specifically suppressed the metastatic ability of these cells. The observed metastasis suppression had no effect on tumor growth rate. Of interest, in analogous studies of human prostate cancer cell lines, transfer of these chromosomes suppressed the cells' tumorigenicity *(69,70)*. These findings could result from at least three alternative mechanisms. First, a given chromosomal region may encode a number of different genes, one or more of which may be active as a tumor-suppressor gene in human prostate cancer cells but be inactive or not expressed in rat prostate cancer cells. Second, genes may function as metastasis-suppressor genes when expressed in rat prostate cancer cells but may be inactive or not expressed in human prostate cancer cell lines. Third, gene(s) that lie in the same chromosomal region may have different functions, depending on the context (i.e., cell type) in which they are expressed.

In the third scenario, the effect of the gene product may be limited or determined by the recipient cells. We refer to this scenario as the "cellular hard-wiring" hypothesis.[1] For example, human prostate cancer cell lines compared with Dunning rat prostate cancer cell lines are weakly metastatic in spontaneous metastasis assays *(62)*. These differences in their *in vivo* biologic activities could be the result of genetic differences between the tumor cells, or they could result from an epigenetic mechanism, such as differential tumor–stromal interactions. The nature of cellular interactions with the extracellular matrix can regulate tissue-specific gene expression, since cells form an elaborate three-dimensional network composed of the nuclear, cytoskeletal, and extracellular matrices *(27,71)*. Thus, the differential effects of a given chromosome transferred into different cell types can be the result of differential expression of the genes on the chromosome as determined by the way a cell responds to its environment.

During the past decade, several human chromosomes have been functionally tested through the use of MMCT, and metastasis-suppressor activities have been reported on chromosomes 1, 6, 7, 8, 10, 11, 12, 16, and 17 *(63,64,72–82)* (Table 1). Such functional studies, combined with positional and expression-based gene cloning techniques, have enabled the identification of KAI1, KISS-1, MKK4/SEK1, and BRMS1 as metastasis-suppressor genes.

Metastasis-Suppressor Genes

As discussed earlier, metastasis-suppressor genes suppress the formation of spontaneous, macroscopic metastases *without affecting the growth rate of the primary tumor.* To date, five genes, nm23 (NME1), KAI1, KiSS1, BrMS1, and MKK4 (MAP2K4), have been shown to meet the criteria of a metastasis-suppressor gene [*see* Table 2; *(31,32,83–119)*]. The role of other genes, such as CD44 and maspin/PI5, in metastasis suppression is less well defined *(102,120–131)*. The potential mechanism of action of all of these genes has been inferred by analogy to other family members and observations in model systems. How these genes and their protein products function to suppress metastasis *in vivo* is the subject of enthusiastic study. Decreased expression of the suppressor gene is the key parameter determining metastatic potential and may occur by a variety of mechanisms, not necessarily LOH *(32,91)*. To date, nm23 (NME1) and KAI1 are the best-characterized metastasis-suppressor genes.

nm23 (NME1)

The prototypical metastasis-suppressor gene, nm23, was identified in the murine K1735 melanoma by use of subtractive hybridization (a method to identify genes differentially expressed between two cell lines), and six human homologues have been identified *(90)*. Loss of Nm23-H1 expression is associated with metastatic potential in many, but not all, late-stage tumors *(91)*. Transfection of nm23-H1 cDNA into highly metastatic murine melanoma, rat mammary adenocarcinoma, and human breast cancer and melanoma cells reduces their invasiveness and metastatic ability *in vivo (91)*. In cancers, such as lung, colon, prostate, etc. [reviewed in *(87)*], where no alterations in the expression pattern of Nm23-H1 are evident, it is possible that the biologic function of Nm23-H1 does not influence malignant progression in these cell types. Alternatively, its effects may be inhibited by alternate mechanisms. The mechanism of action for metastasis suppression by Nm23 still remains unknown; however, evidence suggests that it is phosphorylated and may be involved in a novel signaling pathway that, in turn, controls cell motility *(84,87)*.

KAI1

The localization of metastasis-suppressor activity to rat chromosome 2 in the cell fusion experiments by Ichikawa et al. *(57)* prompted the search for homologous metastasis-suppressor genes for human prostate cancer. The first of such genes identified was KAI1. MMCT was used to transfer human chromosome 11 into Dunning AT6.1 and AT3.1 rat prostate cancer cells, and the resulting microcell hybrids were assayed for metastasis suppression in immunodeficient mice *(81)*. These studies led to the identification of the metastasis-suppressor gene KAI1, which maps to 11p11.2–p13 *(101)*. The metastasis-suppressor activity of KAI1 was subsequently demonstrated by transfecting it into AT6.1 cells and assaying the metastatic ability of individual transfected control cell lines in severe combined immunodeficient (SCID) mice *(101)*. Reports *(101,110)* suggest that expression of KAI1 decreases both the invasiveness and motility of cells *in vitro*. Additional studies show that KAI1 transfectants exhibit enhanced Ca^{2+}-independent aggregation, indicating that KAI1 expression alters cell–cell interactions *(109)*. The metastasis-suppressor activity of KAI1 was subsequently demonstrated by its transfection into AT6.1 cells and assaying the metastatic ability of individual transfected control cell lines in SCID mice *(101)*. Lowered expression of KAI1 has also been associated with progression in a wide variety of cancers, including pancreatic, hepatocellular, bladder, breast, and non-small-cell lung cancers *(31,133–136)*, as well as esophageal cell carcinomas *(137)* and squamous and lymphoid neoplasms *(138)*. These data suggest that KAI1 has a conserved metastasis-suppressor function. Furthermore, these studies demonstrate that metastasis-suppressor genes can be developed as clinical markers *even before their biochemical mechanism of action has been elucidated.*

Emerging Role of Metastasis-Suppressor Genes in the Regulation of Metastatic Growth

While it is tempting to speculate on the mechanism of action of genes shown in Table 2, examination of how genes, such as MKK4 or BRMS1, suppress metastasis will require construction of appropriate biochemical constructs and identification of *in vitro* conditions that will enable us to conduct meaningful biochemical and molecular studies. As a first step to accomplishing this goal, our laboratories have initiated studies designed to examine the step in the metastatic cascade inhibited by a chromosome or gene of interest. As an example of these studies, we will present recent work on the metastasis-suppressor activity encoded by chromosomes 17 and 6. These studies have brought us closer to defining mechanisms of metastasis suppression.

Chromosome 17

We have reported the identification of discontinuous portions of human chromosome 17 (D17S952 → D17S805, D17S930 → D17S797, and D17S944 → qter) that together suppress the metastatic ability of AT6.1 Dunning rat prostatic cancer cells when introduced via MMCT *(63,80)*. PCR and Southern blot analyses demonstrated that three of the four markers on 17p13, including HIC1 and TP53, and 12 of the 13 markers in 17q21-23, including BRCA1 and the metastasis-suppressor gene NME1 (nm23), were not retained in this region *(63)*. AT6.1 microcell hybrids containing this portion of chromosome 17 were tested *in vivo* in spontaneous metastasis assays. Spontaneous metastasis is measured by the ability of tumor cells to form a locally growing tumor at the site of injection and disseminate and grow at to secondary sites thereafter.

At the experimental end point, the number of overt surface metastases observed in the lungs from mice with AT6.1–17 tumors was reduced 15- to 30-fold compared with lungs from mice bearing parental AT6.1 tumors *(63)*. This suppression could be due to the inhibition of any step within the metastatic cascade. We reasoned that examination of the biology of metastasis suppression would provide clues to the identity of genes responsible for suppression of metastatic growth. A series of *in vivo* experiments were conducted, and no evidence was found to suggest that there is a decrease in the number and/or viability of tumor cells colonizing the lung *(80)*.

On the basis of these findings, we hypothesized that a gene or genes encoded by the suppressor region of chromosome 17 function by inhibiting the growth of metastases *in the lung (139)*. To test this possibility, AT6.1–17 cells were transduced with a β-galactosidase reporter gene construct (AT6.1–17Tβgal cells)

Table 2. Summary of metastasis-suppressor genes identified*

Metastasis suppressor gene (reference No.)	Discovery method	Tumor type or site (reference Nos.)	Cell lines transfected†	*In vitro* phenotype†	*In vivo* phenotype	Status in clinical disease	Reported mechanisms of action (reference Nos.)
nm23‡ (NME1) (17q21.3) *(83)*	cDNA subtraction	Melanoma *(84–89)*	K-1735 (mouse)	↓ Motility ↓ Colony formation ↓ Proliferating (TGFβ)	↓ Exp. mets.	Inverse correlation between Nm23 expression and metastatic potential	• Nucleotide diphosphate kinase • Signal transduction • Transcriptional activation *(90,91)*
			B16 F10 (mouse)	↓ Invasiveness ↑ Cell–cell adhesion ↑ Immunosensitivity	↓ Exp. mets.		
			B16 FE7 (mouse)	ND	↓ Exp. mets.		
			MelJuSo (human)	ND	↓ Exp. mets		
		Breast *(84,87,92–95)*	MDA-MB-435 (human)	↓ Motility ↓ Colony formation	↓ Spont. mets.	Inverse correlation between Nm23 expression and metastatic potential	
				ND	↓ Spont. mets.		
		Prostate *(87,96)*	MTLn3 (rat) DU145 (human)	↓ Colony formation ↓ Invasiveness ↓ Adhesion to extracellular matrix components	ND	No trend observed	
		Colon *(87,97)*	HD3§ (human) (AS-oligo study)	↓ Adhesion to tissue culture dish ↓ Growth arrest ↓ Differentiation	ND	Aggressive colorectal cancers have high expression of mutated Nm23	
			U9§ (human) (AS-oligo study)	No change	ND		
		Oral *(98,99)*	LMF4	ND	↓ Exp. mets. ↑ Differentiation	Inverse correlation between Nm23 expression and metastatic potential	
KAI1‖ (11p11.2) also known as CD82 *(100)*	MMCT/Alu-specific PCR/hybridization of cDNA library	Prostate *(32,100–103)*	AT6.1 (rat)	↓ Invasiveness	↓ Spont. mets.	Inverse correlation between protein expression and metastatic potential	• Integrin signaling • Cell adhesion • Motility *(104–106)*
			AT3.1 (rat)	ND	NE spont. mets.		
			AT6.3 (rat)	ND	↓ Spont. mets.		
		Breast *(31,75, 107,108)*	MDA-MB-435 (human)		↓ Spont. mets.	Inverse correlation between protein expression and metastatic potential	
			• ch 11 MCT	↓ Invasiveness	Protein expression/ modification in the primary tumors and mets.		
			• KAI1 cDNA transfection	ND			
		Melanoma *(109)*	MelJuSo (human)	ND	↓ Exp. mets.	ND	
				↑ Cell aggregation	↓ Exp. mets.		
			B16-B16 (mouse)	↓ Motility ↓ Invasiveness			
		Colon *(110,111)*	BM314 (human)	↑ Cell aggregation ↓ Motility ↓ Invasiveness	ND	Inverse correlation between protein expression and metastatic potential	
			DLD-1 (human)	↑ Cell aggregation ↓ Motility ↓ Invasiveness	ND		

(Table continues)

Table 2 (continued). Summary of metastasis-suppressor genes identified*

Metastasis suppressor gene (reference No.)	Discovery method	Tumor type or site (reference Nos.)	Cell lines transfected†	*In vitro* phenotype†	*In vivo* phenotype	Status in clinical disease	Reported mechanisms of action (reference Nos.)
KiSS1 (1q32) *(112)*	MMCT/ cDNA subtraction	Melanoma *(89,113,114)*	C8161	NE adhesion to extracellular matrix components NE invasion	↓ Exp. mets. ↓ Spont. mets.	ND	• Signal transduction *(113)*
			MelJuSo	ND	↓ Exp. mets. ↓ Spont. mets.		
		Breast *(114)*	MDA-MB-435	↓ Colony formation ↓ Spread on collagen type IV NE motility	↓ Spont. mets.	ND	
BrMS1 (11q13.1–2) *(115)*	MMCT/ differential display	Breast *(115)*	MDA-MB-435 (human)	ND	↓ Spont. mets.	ND	• Cell communication • Motility *(115)*
			MDA-MB-231 (human)	ND	↓ Exp. mets.		
MKK4 (MAP2K4) (17p11.2) *(116)*	MMCT/ positional EST identification	Prostate *(117)*	AT6.1 (rat)	ND	↓ Spont. mets.	ND	• Cytokine/ stress-induced signal transduction *(118,119)*
CD44¶ (11p13) *(120)*	MMCT	Prostate *(102,121–126)*	AT3.1 (rat)	ND	↓ Spont. mets.	Decreased expression of CD44 correlates with higher tumor grade, aneuploidy, and presence of distant metastases	• Receptor for both hyaluronic acid and osteopontin • Cell adhesion *(127)*
Maspin¶ (PI5) (18q21.3) *(128)*	Subtractive hybridization differential display	Breast *(129,130)*	MDA-MB-435 (human)	↓ Invasiveness ↓ Motility	↓ Primary tumor growth	ND (no cohort studies, although weak expression in malignant cells of invasive breast carcinomas has been reported)	• Serine protease inhibitor • Modulation of integrin expression *(130)*
		Prostate *(131)*	AT3.1 (rat)	ND	NE primary tumor growth NE spont. mets.	ND	

*TGFβ = transforming growth factor-beta, ND = not determined, MMCT = microcell-mediated chromosomal transfer, cDNA = complementary DNA, NE = not examined, exp. mets. = experimental metastases, spont. mets. = spontaneous metastases, PCR = polymerase chain reaction, AS = antisense, EST = expressed sequence tag.

†Cell motility was determined in chemotaxis assays by use of Boyden chambers, in phagokinetic track assays on coverslips, or by cinematography studies. Invasion was measured by migration through Matrigel or reconstituted basement membranes in Boyden chambers. Colony formation was evaluated in soft agar. Cell proliferation was measured by counting viable cells using a hemocytometer. Cell adhesion was evaluated by the ability of cells to form conjugates with lymphokine-activated killer cells (LAK), the ability to adhere to tissue culture plates coated with laminin, fibronectin, collagen type I, or collagen type IV in the absence of fetal bovine serum (FBS), or by the ability to remain adherent to tissue culture plates after the removal of FBS and the addition of oligonucleotides and TGFβ. Immunosensitivity was determined in chromium-release assays with LAK cells. Cell aggregation was examined by culturing single-cell suspensions in Puck's saline plus 0.8% FBS. Cell spreading over extracellular matrix substrates was monitored over time by photography.

‡Additional clinical studies have examined the expression of Nm23 in hepatocellular, gastric, ovarian, and cervical carcinomas *(87)*.

§HD3 and U9 are sublines of the human colon carcinoma line, HT29, and differ in their responses to TGFβ.

‖Inverse correlations between KAI1 protein and/or messenger RNA expression and malignant potential have been observed in pancreatic, non-small-cell lung, bladder, hepatocellular, and esophageal squamous cell carcinomas.

¶Does not fit the classic definition of a metastasis-suppressor gene.

and tested in spontaneous metastasis assays *(2)*. At the experimental end point, animals were killed and the excised lungs were stained for β-galactosidase expression. This approach allowed the visualization of microscopic AT6.1–17βgal surface metastases. Subcutaneous injection of AT6.1 parental cells resulted in the formation of a mean number of 97 overt surface metastases (detected using Bouin's fixation) per lung (Fig. 3, A; left). As expected, the number of overt macroscopic metastases after the subcutaneous injection of AT6.1–17-Tβgal cells was greatly reduced (Fig. 3, A; middle). In contrast, when lungs removed from the mice carrying AT6.1–17-Tβgal tumors were stained for β-galactosidase activity, numerous blue-staining microscopic

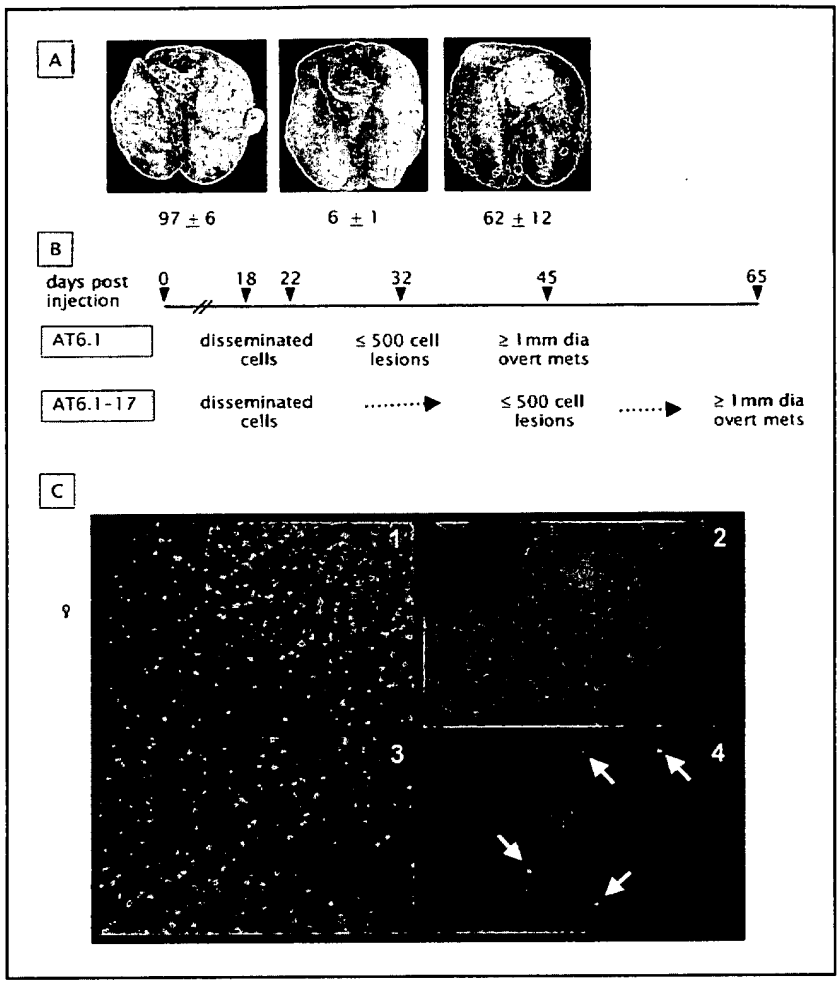

Fig. 3. Examination of the mechanism of metastasis suppression by chromosome 17 and 6. **A)** Quantification of overt surface metastases and micrometastases. AT6.1 cells are highly metastatic rat prostate cancer cells. AT6.1–17-Tβgal cells contain the metastasis-suppressor region of human chromosome 17 and are tagged with a β-galactosidase reporter gene enabling the sensitive detection of microscopic metastases. The numbers of overt and microscopic metastases were determined by use of Bouin's fixation and β-galactosidase activity, respectively. At the experimental end point, lungs were removed from tumor-bearing animals. **Left**—lung from AT6.1 tumor-bearing animal stained with Bouin's solution; **middle**—lung from AT6.1–17-Tβgal tumor-bearing animal stained with Bouin's solution; and **right**—lung from AT6.1–17-Tβgal tumor-bearing animal stained for β-galactosidase activity. The average number of overt or microscopic metastases and standard error are shown **below the panels.** This figure is adapted from *(139).* **B)** A combination of techniques has been used to examine the time course of cancer cell dissemination and growth in suppressed AT6.1–17 cells as compared with metastatic AT6.1 parental cells. These data indicate that genes encoded by chromosome 17 inhibit a step in metastatic colonization. **C)** Photomicrographs of mouse lung following intravenous injection of green fluorescent protein-tagged C8161 and metastasis-suppressed neo6/C8161 cells (**panel C3**) are present. At 1 month, however, C8161 cells have proliferated to form macroscopic lung lesions (**C2**), but most neo6/C8161 cells have been cleared. Occasional single cells (**C4, arrows**) can be found in the lungs but fail to proliferate. These results imply that chromosome 6 suppresses metastasis by inhibiting the ability of C8161 cells to grow in the lung at an early stage of colonization (original magnification ×300). Data adapted from *(144).*

metastases were observed (Fig. 3, A; right). Of interest, the mean number of AT6.1–17-Tβgal micrometastases (i.e., 62 ± 12 standard error [SE]) detected by this method is on the same order of magnitude as the mean number of macroscopic AT6.1 metastases (i.e., 97 ± 6 SE). These results demonstrate that AT6.1–17 cells do escape from the primary tumor and arrive in the lungs but do not form large metastatic foci *(139).* Development of overt metastases was associated with loss of the metastasis-suppressor region of chromosome 17 *(139).*

Because of the similarity between our findings to the angiostatin-mediated dormancy reported by Holmgren et al. *(140),* we investigated the possibility that AT6.1–17 primary tumors secrete a substance that suppresses the growth of its own metastases *(139).* For this experiment, 2×10^5 AT6.1–17 cells were injected subcutaneously into the flanks of SCID mice, which were then divided into two experimental groups. Once the tumors reached a volume of 1 cm^3, they were surgically removed from the mice in the first group, while those in the second group were left intact, although a contralateral sham surgery was performed. It was anticipated that if the AT6.1–17 primary tumor secreted a substance like angiostatin, which suppresses the growth of its own metastases, then a substantial increase in the number of overt metastases should develop in the lungs of mice in which the primary tumors had been removed. However, after approximately 65 days after injection, the animals were killed and examination of the lungs from both groups showed no difference in the numbers of overt macrometastases *(139).* Thus, these studies found no evidence for an antiangiogenic mechanism in this model.

Taken together, our data suggested that AT6.1–17 cells escape from the primary tumor but are growth inhibited at the secondary site *(139).* If this is an early event, we predicted that viable, disseminated AT6.1 and AT6.1–17 cells should be present in the lung at very early time points. We found that viable cells could be harvested from the lungs of both AT6.1 and AT6.1–17 tumor bearers as early as 18 days after injection (Fig. 3, B). Our preliminary time-course data show that AT6.1–17 cells disseminate and lodge in the lungs but have an extended latent period as compared with AT6.1 parental cells.

Chromosome 6

On the basis of the high incidence of chromosome 6 abnormalities in late-stage human melanoma *(141),* we introduced an intact chromosome 6 into the highly metastatic C8161 human melanoma cells by MMCT. Parental cells formed tumors in every mouse given an intradermal injection of 1×10^6 cells, and more than 90% of the mice developed regional lymph node and lung metastases. In contrast, chromosome 6-C8161 hybrids (neo6/C8161) were still tumorigenic but completely suppressed for metastasis *(142).* Intravenous injection of neo6/C8161 cells also did not produce metastases. In a recent study *(143),* introduction of a version of a chromosome 6 with deletions on the

long arm allowed refinement of the metastasis-suppressor locus to a 40-megabase (Mb) region represented by chromosomal bands 6q16.3–q23.

The mechanism of action for the metastasis-suppressor protein from the gene on chromosome 6 was studied using a variety of *in vitro* and *in vivo* techniques. The neo6/C8161 cells were still locally invasive, and cells were even detected in efferent vessels. This finding implied that the step(s) in the metastatic cascade inhibited by introduction of chromosome 6 occurred subsequent to intravasation. The identity of those steps was not further elucidated using *in vitro* assays mimicking adhesion, invasion, motility, or growth. No important differences between the metastatic and nonmetastatic cells were observed using the many *in vitro* assays *(72,73,141,142)*.

For a better definition of the step(s) in metastasis blocked by addition of chromosome 6, cells that constitutively express GFP were engineered. GFP-tagged C8161 and neo6/C8161 cells were injected intravenously into athymic mice. C8161, as expected, formed overt metastases, but neo6/C8161 cells did not. Microscopic metastases (single cells or clusters of <10 cells) were observed in the lungs following neo6/C8161 cell injection, suggesting that these cells lodged in the lungs but failed to proliferate *(144)*. For the determination of whether the fluorescing cells were viable, they were isolated from the lung up to 60 days after injection and grown in culture. On injection into the skin of athymic mice, the neo6/C8161 cells isolated from the lung grew at rates similar to those of previously injected neo6/C8161 cells. This result implies that the gene or genes on chromosome 6 interfere specifically with growth-regulatory responses in the lung but not in the skin.

From Gene Discovery to Clinical Utility

This review has focused on the identification and development of metastasis-suppressor genes as new additions to our molecular armamentarium. As translational researchers, our immediate goals are 1) to improve the ability of the pathologist to distinguish unambiguously malignant from indolent lesions and 2) to help the clinician differentiate tumors that are highly likely to metastasize from those that are not. The practical question, therefore, is: How can we use these genes, or the pathways that they regulate, to improve patient management? When the search for metastasis-suppressor genes was initiated in the late 1980s, the major challenge was the identification of candidate genes. Recently, however, there has been an explosion in the genetic information that is instantly available. Furthermore, because of the efforts of independent laboratories and cooperative efforts, such as the Cancer Genome Anatomy Project of the National Cancer Institute (Bethesda, MD), cancer transcriptomes and proteomes will soon be available *(145,146)*. New technologies will continue to increase our ability to dissect molecular pathways in individual cells within human cancers. While this wealth of information will no doubt be of use, work from the groups of Bissell, Cunha, and Chung *(147–154)* has clearly demonstrated that tissue structure determines, or at least greatly influences, gene expression and function. Thus, it may be extremely difficult to predict the importance of genes expressed in individual microdissected cancer cells to the biology of the intact tumor, the behavior of which is determined by complex interactions among a population of cells. The present challenge is to identify the genes that are *functionally important* in the acquisition of metastatic ability. Achieving this goal will require the use of well-characterized, *in vivo* (animal) models coupled with clinical correlative studies. It must be emphasized that *in vitro models do not accurately* reflect *in vivo* metastasis *(155)*. *Indeed, none of the metastasis-suppressor genes described herein could have been identified using traditional in vitro assays.* Given the inherent variability and nonlinear behaviors of biologic systems, it is probable that no one model will prove to be adequate to separate out the contributions of the multiplicity of genes involved in the development of metastases. Thus, it is more advantageous to focus studies on a particular model and tease out important cellular pathways modulated by a particular gene of interest in that model and then to test and verify the importance of the target pathway in clinical disease as well as in additional model systems.

Technologic advances are enabling us to examine the metastatic process and the genes that regulate it in new ways. This ability has led us to re-evaluate fundamental concepts concerning the determinants of metastatic propensity. In the past, the escape of cells from the primary site was viewed as the rate-limiting step for the development of metastases. The clinical implication was that disseminated cancer cells were destined to grow into lethal metastases; thus, they were not a target for therapeutic intervention *(18)*. Findings from clinical studies and basic research from several independent laboratories have shown that survival and subsequent growth of extravasated cancer cells at the secondary site may determine metastatic efficacy. These observations are driving our laboratories and others to reconsider the role of endothelial cell–tumor cell interactions in survival, signaling, and growth control cascades to develop new strategies for controlling the growth of disseminated cancer cells *(39,45,156)*.

As metastasis researchers, we find ourselves in the midst of a revolution. In preparing this review, we considered the parallels between recent developments in our field and the development of the field of molecular biology. Much of early molecular biology was pursued by individuals who were not trained as biologists, but as physicists, such as Max Delbrück *(157)*. We are respectful of the observations of Erwin Schrödinger, the father of statistical mechanics, who observed that, "all of the physical and chemical laws that are known to play an important part in the life of organisms are of the statistical kind. The behavior of such systems depends entirely on a large number of molecules that cooperate to form the observed function or phenotype" *(158)*. Although this comment was made in regard to normal biologic processes, it is equally applicable to the multiple genetic changes that are required for the acquisition of metastatic ability. Metastasis is a complex, multigenic phenotype. As such, multiple markers will be needed for the accurate assessment of the metastatic ability of tumors and tumor cells. This need is highlighted by the tremendous impact of seemingly trivial experimental manipulations on the outcome of metastasis assays *(155)*. Parallels have been drawn between the behavior of cancer cells and complex adaptive systems *(159,160)*. As such, very small changes in initial conditions may produce an outcome of such great diversity as to appear random *(159)*. Ultimately, we believe that, to translate our molecular findings into meaningful markers, we will have to go beyond our traditional areas of expertise and work with mathematicians, computational biologists, and others to take this revolution from bench to bedside.

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NOTES

[1]In our work, the concept of cellular hardwiring refers to the work of Pienta and Coffey *(71)* and Pienta et al. *(132)*.

Supported by Public Health Service (PHS) grant CA62168 (D. R. Welch) from the National Cancer Institute (NCI), National Institutes of Health (NIH), Department of Health and Human Services (DHHS); by DAMD1-96-6152 (D. R. Welch) from the Army Medical Research and Materiel Fund; by The National Foundation for Cancer Research (D. R. Welch); by The Jake Gittlen Memorial Golf Tournament (D. R. Welch); by PHS grants P20CA66132 and R29CA6948702 (C. W. Rinker-Schaeffer) from the NCI, NIH, DHHS; by Cancer Research Foundation Young Investigator Award (C. W. Rinker-Schaeffer); by the American Foundation for Urologic Disease (B. A. Yoshida and C. W. Rinker-Schaeffer), and by the University of Chicago RESCUE Fund (B. A. Yoshida, C. W. Rinker-Schaeffer).

We thank Ms. Zita Dubauskas and Mr. Edwin F. Schaeffer III for their editorial efforts. We also thank Dr. Charles Brendler of the University of Chicago for his enthusiastic support of this research.

Manuscript received January 7, 2000; revised August 15, 2000; accepted August 29, 2000.

☐ 1: Nat Genet. 1997 Jun;16(2):133-43. Related Articles, Links

Comment in:
- Nat Genet. 1997 Jun;16(2):113-4.

Functional expression and germline transmission of a human chromosome fragment in chimaeric mice.

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Human chromosomes or chromosome fragments derived from normal fibroblasts were introduced into mouse embryonic stem (ES) cells via microcell-mediated chromosome transfer (MMCT) and viable chimaeric mice were produced from them. Transferred chromosomes were stably retained, and human genes, including immunoglobulin (Ig) kappa, heavy, lambda genes, were expressed in proper tissue-specific manner in adult chimaeric tissues. In the case of a human chromosome (hChr.) 2-derived fragment, it was found to be transmitted to the offspring through the germline. Our study demonstrates that MMCT allows for introduction of very large amounts of foreign genetic material into mice. This novel procedure will facilitate the functional analyses of human genomes in vivo.

PMID: 9171824 [PubMed - indexed for MEDLINE]

Double trans-chromosomic mice: Maintenance of two individual human chromosome fragments containing Ig heavy and κ loci and expression of fully human antibodies

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Edited by Leroy Hood, University of Washington, Seattle, WA, and approved November 19, 1999 (received for review July 20, 1999)

The use of a human chromosome or its fragment as a vector for animal transgenesis may facilitate functional studies of large human genomic regions. We describe here the generation and analysis of double trans-chromosomic (Tc) mice harboring two individual human chromosome fragments (hCFs). Two transmittable hCFs, one containing the Ig heavy chain locus (*IgH*, ≈1.5 Mb) and the other the κ light chain locus (*Igκ*, ≈2 Mb), were introduced into a mouse strain whose endogenous *IgH* and *Igκ* loci were inactivated. In the resultant double-Tc/double-knockout mice, substantial proportion of the somatic cells retained both hCFs, and the rescue in the defect of Ig production was shown by high level expression of human Ig heavy and κ chains in the absence of mouse heavy and κ chains. In addition, serum expression profiles of four human Ig γ subclasses resembled those seen in humans. They mounted an antigen-specific human antibody response upon immunization with human serum albumin, and human serum albumin-specific human monoclonal antibodies with various isotypes were obtained from them. These results represent a generation of mice with "humanized" loci by using the transmittable hCFs, which suggest that the Tc technology may allow for the humanization of over megabase-sized, complex loci in mice or other animals. Such animals may be useful not only for studying *in vivo* functions of the human genome but also for obtaining various therapeutic products.

Technical advances that enable larger stretches of human DNA to be introduced into mice allow not only for introduction of large genes or gene clusters but also correct expression of transgenes by inclusion of essential remote regulatory elements (1). This also facilitates the generation of mice with "humanized" loci whose endogenous loci are functionally substituted for intact human equivalents in combination with targeted inactivation of endogenous loci, thereby providing valuable experimental animals for gaining insight into *in vivo* functions of human genes and for studying human genetic disorders (2, 3). Particularly, much effort has been made by a number of groups to create mice with humanized Ig (*Ig*) loci for obtaining therapeutic human mAbs (hu-mAbs) monoclonal antibodies (4, 5). Their studies established that transgenic mice carrying a portion of human *IgH* (14q32.33, ≈1.5 Mb) and *Igκ* (2q12, ≈2 Mb) loci in the endogenous *Ig*-knockout (KO) background were successfully used for the production of antigen-specific fully human antibodies. They also showed that the use of larger transgenes containing a larger number of V-gene segments resulted in mice exhibiting more efficient humoral response to a wide range of antigens. Although the introduction of entire human *Ig* loci into mice to reconstitute full diverse human antibody repertoires has been a next major challenge, this has never been achieved because the cloning of over megabase-sized DNA fragments encompassing whole human *Ig* loci remains difficult even with the use of yeast artificial chromosomes (1, 5). In addition, the constant region of the human *IgH* locus is known to contain sequences difficult to be cloned (6).

To circumvent such a DNA cloning step, we have developed a procedure utilizing a human chromosome fragment (hCF) as a vector for transgenesis. In our previous study (7), various hCFs were introduced into mouse embryonic stem (ES) cells via microcell-mediated chromosome transfer, and viable chimeric mice were produced from them. Transferred hCFs were stably retained, and human genes, including the Igκ, heavy, and -λ genes, were expressed in a proper tissue-specific manner in adult chimeric tissues. In the case of a human chromosome 2 (hChr.2)-derived hCF [hCF(2-W23), ≈5–20 Mb] (8) containing the *Igκ* locus, it was found to be transmitted to the offspring through the germ line, demonstrating the establishment of a trans-chromosomic (Tc) mouse [Tc(W23)] expressing the human Ig κ light chain (hκ) (7). Another group also employed the microcell-mediated chromosome transfer to produce chimeric mice containing a hChr.21 or its fragment in a recent report (9).

This procedure was anticipated to be used to generate mice with humanized *Ig* loci; however, several issues remained to be explored to attain this goal. For example, the somatic mosaicism and the transmission efficiency of hCF(2-W23) were not evaluated, and the hChr.14-derived hCF (>50 Mb) containing the *IgH* locus was not transmittable (7). In the present study, we therefore examined: (*i*) germ-line transmission of another hCF with the *IgH* locus, (*ii*) stability of hCFs in the somatic cells, (*iii*) transmission efficiency and functional stability of hCFs during several passages through the germ line, and (*iv*) functioning of the two individual hCFs in mice. Indeed, such studies are prerequisite to the generation of mice containing four distinct genetic modifications (*IgH*-Tc, *Igκ*-Tc, *IgH*-KO, *Igκ*-KO) and expressing fully human Ig molecules comprising human Ig heavy and κ light chains. They are also crucial to demonstrate that the Tc technology can be generally used for humanizing the mouse genome.

Here, double-Tc/double-KO mice expressing fully human antibodies have been successfully generated by the establishment of a Tc strain that retains a hChr.14-derived hCF with the *IgH* locus, and the breeding of this strain with the Tc(W23) strain on a *IgH*- and *Igκ*-KO background. ELISAs showed the high level expression of

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: Tc mouse, trans-chromosomic mouse; hCF, human chromosome fragment; KO mouse, knockout mouse; HSA, human serum albumin; hu-mAbs, human monoclonal antibodies; ES cells, mouse embryonic stem cells; hChr.2, human chromosome 2; MH(ES) cells, microcell-hybrid ES cells; FISH, fluorescence *in situ* hybridization.

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human Igμ, all four Igγ subclasses, and Igκ in the sera of resultant double-Tc/KO mice. Furthermore, hybridomas secreting antigen-specific hu-mAbs with various isotypes were obtained from them, indicating the reconstitution of a functional repertoire of fully human Igs. A description regarding the *in vivo* stability of hCFs is also provided.

Materials and Methods

Genomic DNA Analysis by PCR. Reaction conditions for PCR amplification and the primer sequence of markers except for AKT1 were described in our previous report (7) and The Genome Database (http://www.gdb.org). Seven markers for the primary analysis of A9/SC20 were NP, MYH6, D14S75, D14S66, D14S43, D14S78, and IGHMC (IgM). D14S826, D14S1419, and D14S1420 were mapped within 200 kb of the telomere of chromosome 14q, which is at the 5′ end of *IgH* locus (10). D14S543 and AKT1 are mapped 14q32.1–32.2 and 14q32.32, respectively (http://www.gdb.org). The AKT1 primer pair was 5′-ACGGGCACATTAAGATCACA-3′, 5′-TGCCGCAAAAGGTCTTCATG-3′.

Generation of Tc Mice. The microcell-mediated chromosome transfer, ES cell manipulation and chimera production were carried out as described (7).

Fluorescence *in Situ* Hybridization (FISH) Analysis. Preparation of chromosome samples and FISH analysis were carried out essentially as described (7). Probes are as follows: digoxigenin (Boehringer Mannheim)-labeled human COT-1 DNA (BRL), digoxigenin-labeled 14/22cen (hChr.14/22 α-satellite, Oncor), biotin-labeled 14qter (ID Labs Biotech, Biotechnology, ON, Canada), digoxigenin-labeled mouse major satellite (11), and digoxigenin-labeled mouse minor satellite (11). For two-color FISH analysis, digoxigenin-labeled 14/22cen (Oncor) and biotin-labeled 2cen (hChr.2 α-satellite, Oncor) probes were used. Digoxigenin- and biotin-labeled probes were detected with anti-digoxigenin-rhodamine (Boehringer Mannheim) and FITC-avidin (Vector Laboratories), respectively. Fibroblasts prepared from tail of 4- to 6-week-old mice were cultured in DMEM containing 10% FCS for 2 weeks and then were used for preparation of metaphase chromosomes and interphase nuclei. Fifty metaphase spreads were scored to determine somatic mosaicism of each hCF. Mitotic stability of hCFs in ES cells was determined as follows. The clone #21 and microcell-hybrid ES [MH(ES)] 2-21 (7) were cultured for a week in the presence of 300 μg/ml G418 (GIBCO/BRL) and 0.75 μg/ml puromycin (Sigma), respectively. Then the plate was split 1/8 into a 35-mm plate and was grown to 80% confluency (2 days) in the media lacking the drugs. This process was repeated for 45 days. The retention of the hCF in the cells sampled at day 0, day 30, and day 45 was analyzed by FISH using the COT-1 probe. At each time point, 50 metaphase spreads were scored. The loss rate of each hCF was calculated in three independent experiments and was averaged.

Generation of Ig KO Strains. Detailed protocol for generating *IgH*- and *Ig*κ-KO strains is described elsewhere (K.T. and I.I., unpublished work). The essential genetic modification of each strain is as follows. In the *IgH*-KO ($\Delta H^{-/-}$) strain, a *Bam*HI-*Xho*I genomic segment (3.7 kb) including a portion of Cμ2, Cμ3-Cμ4, and Mμ1-Mμ2 exons was replaced by a neo^r cassette. Absence of Igμ and -γ chain expression in the sera and B220$^+$ cells in peripheral blood mononuclear cells in the homozygotes ($\Delta H^{-/-}$) was confirmed (data not shown; see Fig. 2). In the *Ig*κ-KO strain ($\Delta\kappa^{-/-}$), a *Sac*II-*Bgl*II segment (2 kb) including the Cκ exon was replaced by a neo^r cassette. Absence of the Igκ light chain expression in the mice that lack Cκ exon was reported previously (12). The $\Delta H^{-/-}$ and $\Delta\kappa^{-/-}$ strains were intercrossed to obtain double-KO ($\Delta H^{-/-}$, $\Delta\kappa^{-/-}$) strain. The double-KO strain has been maintained on a mixed background of C57BL/6, CBA, and MCH(ICR). To isolate $\lambda 1^{low}$ mutants, CD-1 stocks obtained from Charles River Breeding Laboratories (Tokyo) were examined by Southern blots (13). Approximately half of the tested CD-1 animals were found to be $\lambda 1^{low}$ homozygotes, and they were bred with double-Tc/KO animals to generate double-Tc/KO($\lambda 1^{low/low}$) strain.

FACS Analysis. Peripheral blood mononuclear cells were prepared from 12-week-old Tc(SC20);$\Delta H^{-/-}$ and $\Delta H^{-/-}$ mice, were treated with Fc Block (PharMingen), were stained with antibodies, and were analyzed on a FACS vantage (Becton Dickinson, CELL QUEST software). Antibodies used were FITC anti-human IgM (PharMingen); phycoerythin (PE) anti-B220 (PharMingen).

ELISAs, Immunization, and Hybridoma Production. Hμ, hγ, and hκ were assayed as described (7). Mλ, human Igα (hα), and Igε (hε) were assayed by using anti-mλ (Caltag, South San Francisco, CA), anti-hα (α1 + α2, Kirkegaard & Perry Laboratories), and anti-hε (PharMingen) immobilized on the plate and detected with peroxidase-conjugated anti-mλ (Caltag), peroxidase-conjugated anti-hα (Kirkegaard & Perry Laboratories), and alkaline phosphatase-conjugated anti-hε (PharMingen), respectively. Similarly, mμ and mγ were assayed by using anti-mμ (Kirkegaard & Perry Laboratories), anti-mγ (Sigma) for capture and anti-mμ (Kirkegaard & Perry Laboratories), anti-mγ (Caltag) for detection, respectively. Mouse IgG3/λ (Sigma), human IgA (Athens Research & Technology, Athens, GA), human IgE (Chemicon), mouse IgM (Sigma), and mouse IgG (PharMingen) were used as standards. The samples, standards, and antibody conjugates were diluted with mouse serum (Sigma)-supplemented PBS. Human γ chain subclasses were assayed by using a Human IgG Subclass Profile ELISA Kit (Zymed).

Mice were immunized twice with human serum albumin (HSA) (Sigma, 50 μg/injection) in Titer Max Gold (CytRx, Norcross, GA) subcutaneously (day 0, day 21). A final intraperitoneal injection of 50 μg of HSA in PBS was given at day 34, three days before fusion. Serum samples, collected at approximately weekly intervals, were diluted 1:1,800, and antigen-specific ELISAs were performed on HSA-coated plates. The presence of HSA-specific human antibody was detected with horseradish peroxidase-conjugated antibodies specific for hγ (Sigma), hμ (Southern Biotechnology Associates), hκ (Southern Biotechnology Associates), and mλ (Caltag). To produce hybridomas, splenocytes from immunized mice were fused with SP2/0-Ag14 myeloma cells by using PEG4000 (Merck). After 14 days the supernatants from hybrids growing in G418 (1 mg/ml) selection medium were first screened for the presence of HSA-specific hμ and hγ. The resulting HSA-specific hγ^+ hybridomas were then assayed by using peroxidase-conjugated antibody specific for hκ, mλ, hγ1 (Zymed), hγ2 (Zymed), hγ3 (Zymed), and hγ4 (Zymed). TMB (Sumitomo bakelite) or BCIP (Kirkegaard & Perry Laboratories) was used for substrates, and the absorbance at 450 nm or 630 nm was measured by using a spectrophotometer (Bio-tek Instruments, Luton, U.K.).

Breeding Analysis. Chimeras and Tc mice were mated with MCH(ICR), mice and the resultant offspring were examined by PCR and ELISAs [hμ for Tc(SC20), hκ for Tc(W23)]. PCR markers used were D14S543 and IGHMC for Tc(SC20); D2S1331 and IGKC for Tc(W23). MCH(ICR) and C57BL/6N mice were purchased from Japan Crea (Tokyo).

Results and Discussion

Generation of Tc Mice Containing the Human *IgH* Locus. A microcell-hybrid mouse A9 cell line, A9/SC20, was a subclone isolated from A9/14-C11 (7), which retained a G418^r-tagged hCF whose size was estimated to be approximately one-fifth of an intact hChr.14 [hCF(SC20), see Fig. 1*A*] and slightly larger than that of the hCF(2-W23). Of seven PCR markers of hChr.14 that were examined, only IgM was detected in this hybrid. Further analysis revealed that it also contained three markers (D14S826, S1419, S1420) residing within the most distal portion of the *IgH* locus (10) and two

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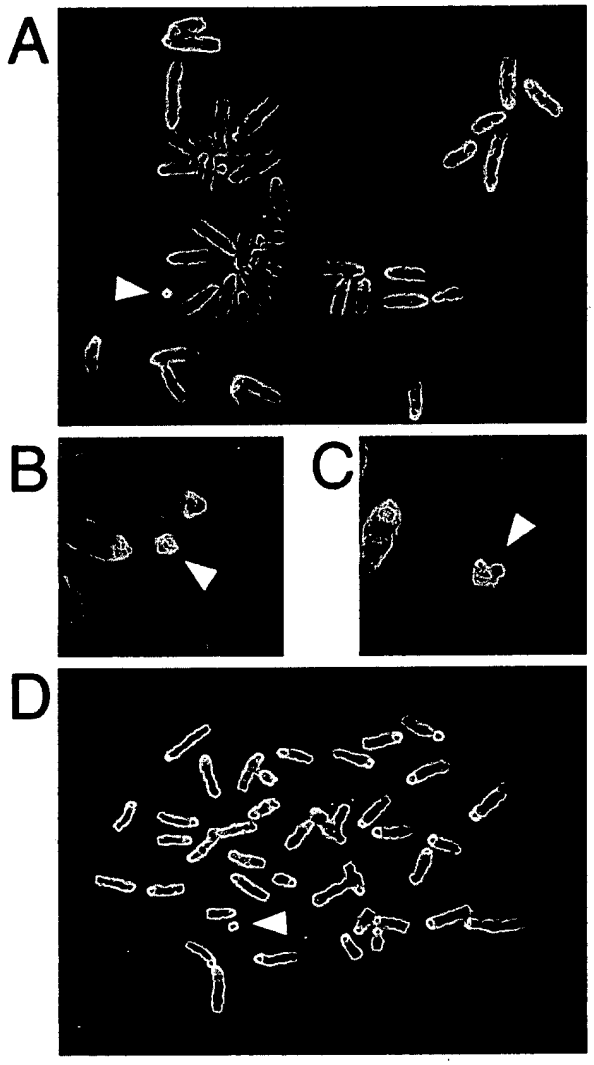

Fig. 1. FISH analysis of MH(ES) clone (#21) and tail fibroblasts prepared from a Tc(SC20) mouse. Shown are photomicrographs of representative metaphase spreads from clone #21 hybridized to human COT-1 DNA (*A*), 14/22cen (*B*, partial spread), or 14qter (*C*, partial spread) probes. Hybridization signals (red, arrowhead) were detected on the transferred hCF(SC20) in mouse chromosomes stained with DAPI (blue). (*D*) Metaphase spreads from tail fibroblasts of a Tc(SC20) animal hybridized to human COT-1 DNA. An extra hCF with similar size to that in the clone #21 (see *A*) was detected (red, arrowhead).

proximal markers (D14S543, AKT1), suggesting that the hCF(SC20) included the whole human *IgH* locus. The hCF(SC20) was introduced into a female mouse ES cell line, TT2F(39,XO), by microcell-mediated chromosome transfer (7) to generate G418^r MH(ES) clones retaining the transferred hCF as an independent chromosome (Fig. 1*A*). Stability tests under the nonselective condition using one of the MH(ES) clones (#21) revealed that the hCF(SC20) was highly stable in mouse ES cells (<0.1% loss/doubling) in contrast to the hCF(2-W23) (3.2% loss/doubling) and the hChr.Y-derived minichromosome reported recently (14). Structural analyses of the hCF(SC20) by FISH showed the absence of murine centromeric sequences (major and minor satellites) (data not shown) and the presence of 14cen (Fig. 1*B*) and 14qter (Fig. 1*C*) sequences, indicating that it was generated as a consequence of an interstitial deletion between 14cen and 14q32-qter regions. Therefore, the human centromeric sequence of the hCF(SC20) is likely to be sufficient for stable maintenance in mouse ES cells.

Table 1. Breeding analysis of Tc mice

	Sex	No. of tested Tc mice	No. of pups*	No. of hCF$^+$	Transmission efficiency, %†
Tc(SC20)					
Chimera-F1‡	f	2	30	10	33
F1-F2	m	3	142	46	32
	f	4	60	20	33
F2-F3	m	2	74	25	34
	f§	5	42	16	38
Tc(W23)					
Chimera-F1	m	1	25	3	12
	f	4	67	22	33
F1-F2	m	8	324	25	8
	f	6	148	32	22
F2-F3	m	8	346	30	9
	f	8	202	48	24

m, male; f, female.

*In the case of chimeras, only ES-derived agouti offspring were examined.

†The expected transmission efficiency in chimeras and Tc mice hemizygous for the hCF is 50% when mitotic stability of the hCF is perfect and it can properly segregate in meiosis. Because 62 and 25 cell divisions are required for generation of mature sperm and oocyte, respectively (26), in mice, expected efficiencies in Tc(W23), deduced from the mitotic loss rate in (MH)ES cells (3.2% loss/doubling), are 7% in male and 22% in female. These figures are very consistent with those observed in the Tc(W23). On the other hand, the difference of transmission efficiency between male and female in the Tc(SC20) is unclear.

‡Because male MH(ES) cells retaining the hCF(SC20) were not generated, only female chimeras that contained it were used in this study.

§The Tc(SC20) F2 female mice were mated with C57BL/6N male.

Twenty phenotypically normal chimeras (15–100% agouti coats) expressing human Igμ (hμ, 2.1–26 mg/liter) and γ (hγ, 0.2–8.3 mg/liter) heavy chains in the sera were obtained from the clone #21, indicating that the hCF(SC20) included a functional human *IgH* locus. The mating of two female chimeras (100% chimerism) with albino MCH(ICR) males resulted in 30 agouti F1 offspring with normal external appearance. Retention of the hCF(SC20) was confirmed in 10 of 30 (33%) offspring by PCR (IgM and D14S543), ELISA (hμ, 2.4–12.8 mg/liter), and FISH (Fig. 1*D*), demonstrating the establishment of a second Tc strain, Tc(SC20). These results suggest that no apparent structural and functional change of the hCF(SC20) occurred during germ-line transmission. Further crossing revealed that the hCF(SC20) could be transmitted through the male germ line (see Table 1).

Stability of hCFs *in Vivo*. Metaphase spreads of tail fibroblasts prepared from the Tc(SC20) and Tc(W23) mice were examined by FISH to evaluate the stability of hCFs in somatic cells. The percentage of the spreads containing the hCF averaged 78 ± 13% (n = 6) and 30 ± 11% (n = 3) in Tc(SC20) and Tc(W23), respectively. Next, we carried out breeding analyses to determine transmission efficiencies of these hCFs through the male and female germ line, which represent their stability in germ cells (Table 1). These results showed that each hCF was retained in a significant proportion of somatic and germ cells in both Tc mice. The overall stability of the hCF(2-W23) was lower than that of the hCF(SC20) in both somatic and germ cells, which may reflect the mitotic instability of the hCF(2-W23) that was observed in the cultured MH(ES) cells. On the other hand, the moderate loss of the hCF(SC20) was unexpected because of its perfect stability in the ES cells. Although many factors might affect mosaicism of the transferred hCFs in the somatic and germ cells, one possible explanation is that the expression of some human genes included in the hCF(SC20) might confer a selective disadvantage to the cells retaining the hCF in mice.

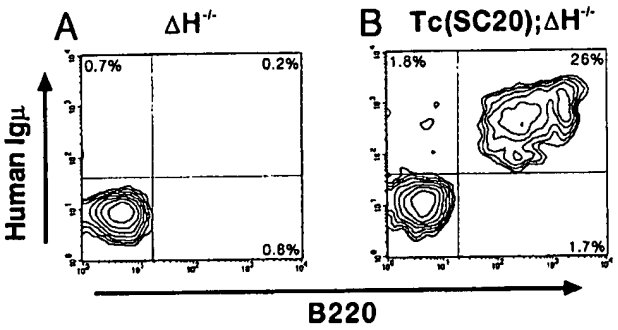

Fig. 2. Rescue of B-cell development in $\Delta H^{-/-}$ mice by introduction of hCF(SC20). Peripheral blood mononuclear cells from $\Delta H^{-/-}$ mouse (*A*) and Tc(SC20);$\Delta H^{-/-}$ mouse (*B*) were assayed for surface expression of hμ and B220. Representative FACS results using 3-month-old animals are shown. The net percentage of positively stained cells is given in each quadrant.

Genetic Rescue of *IgH*-KO Mice by Introducing the hCF(SC20). The Tc(SC20) was bred with the *IgH*-KO ($\Delta H^{-/-}$) strain in which functional B-lymphocytes and Ig production are absent (Fig. 2*A*) to examine whether the introduction of hCF(SC20) with the human *IgH* locus could rescue its phenotypes. As a result, reconstitution of mature B cells ($B220^+$, $h\mu^+$) was observed in peripheral blood lymphocytes prepared from the Tc(SC20);$\Delta H^{-/-}$ mouse (Fig. 2*B*). ELISA analyses also showed the restoration of the serum Igs with hμ or hγ (data not shown; see Fig. 4*A*). Thus, the introduction of hCF(SC20) rescued defects in the *IgH*-KO strain, indicating that the stability of the hCF(SC20) is likely to be sufficient for its persistence in the B cells of adult mice. This demonstrates that the extrachromosomally maintained transgene rescues genetic defects in mice, which may have an implication on the development of mammalian artificial chromosome vectors for gene therapy (15).

Generation of Double-Tc Mice. To obtain mice producing fully human Igs in the absence of endogenous mouse Ig heavy and κ light chains, the Tc(W23) and Tc(SC20) were bred together with the double-KO homozygous strain whose endogenous *IgH* and *Igκ* loci were deleted in both alleles. Screening of 720 offspring obtained from the crossing of Tc(SC20);($\Delta H^{+/-}$, $\Delta\kappa^{+/-}$) and Tc(W23);($\Delta H^{+/-}$, $\Delta\kappa^{+/-}$) animals, by PCR [IgM and D14S543 for hCF(SC20), IGKC and D2S1331 for hCF(2-W23)] and ELISA (hμ and hκ) analysis, showed that 58 individuals were double-Tc that contained all of the PCR markers and produced both hμ and hκ in the sera (data not shown). Two-color FISH analysis of tail fibroblasts prepared from the double-Tc animals by using hChr.2- and hChr.14-specific probes (Fig. 3) showed the retention of each hCF as an independent chromosome. Average mosaicism of the hCF(SC20) and hCF(2-W23) in metaphase spreads were 75 ± 10% and 32 ± 13% ($n = 4$), respectively, similar to those in each Tc strain with a single hCF as described in the text. The percentage of the spreads containing both hCFs averaged 21 ± 7%, which is in good agreement with that deduced from the mosaicism of each hCF (75% × 32% = 24%). Thus, two individual, transferred hCFs could be retained and function in the mouse. Preliminary breeding analysis showed that both hCFs could be transmitted through the germ line of male and female double-Tc animals and that the transmission efficiency of each hCF was similar to that in single-Tc animals (data not shown; see Table 1).

Expression of Human Igs in Double-Tc/double-KO Mice. Double-Tc mice with the double-KO background (double-Tc/KO) were identified by Southern blots (data not shown) and were examined by ELISAs to determine serum concentrations of human Igs. Compared with the $\Delta H^{+/-}$ mice kept under similar conditions, the average levels of hμ and hγ were equivalent to the mouse μ chain (mμ) level and a half of the mouse γ chain (mγ) level, respectively (Fig. 4*A*). All four hγ subclasses were produced and, interestingly, the average proportion of each subclass relative to total hγ concentration was similar to that observed in human serum (Fig. 4*B*). This suggests that the regulatory mechanism determining the expression level of each hγ subclass is similar between human and mouse. Human Igα (α1 + α2, 79–502 μg/liter) and -ε (13–97 μg/liter) chains were also detectable in 8 and 6 of 10 tested

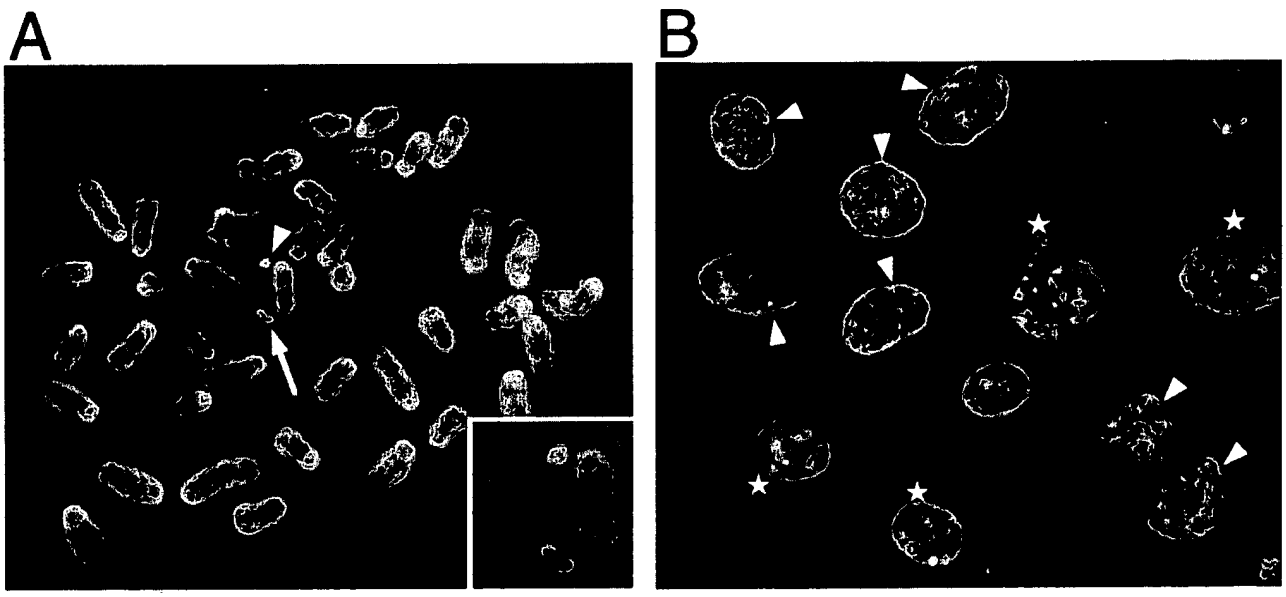

Fig. 3. Two-color FISH analysis of tail fibroblasts prepared from a 4-week-old double-Tc mouse. (*A*) Metaphase spreads were hybridized to 14/22cen (red, arrow) and 2cen (green, arrowhead) probes to detect hCF(SC20) and hCF(2-W23), respectively. A photomicrograph of a representative spread is shown. A magnified (×2) image of the hCFs is provided in the inset. (*B*) Interphase nuclei were hybridized to 14/22cen (red) and 2cen (green) probes. Nuclei containing both signals and a red signal only are indicated with stars and arrowheads, respectively.

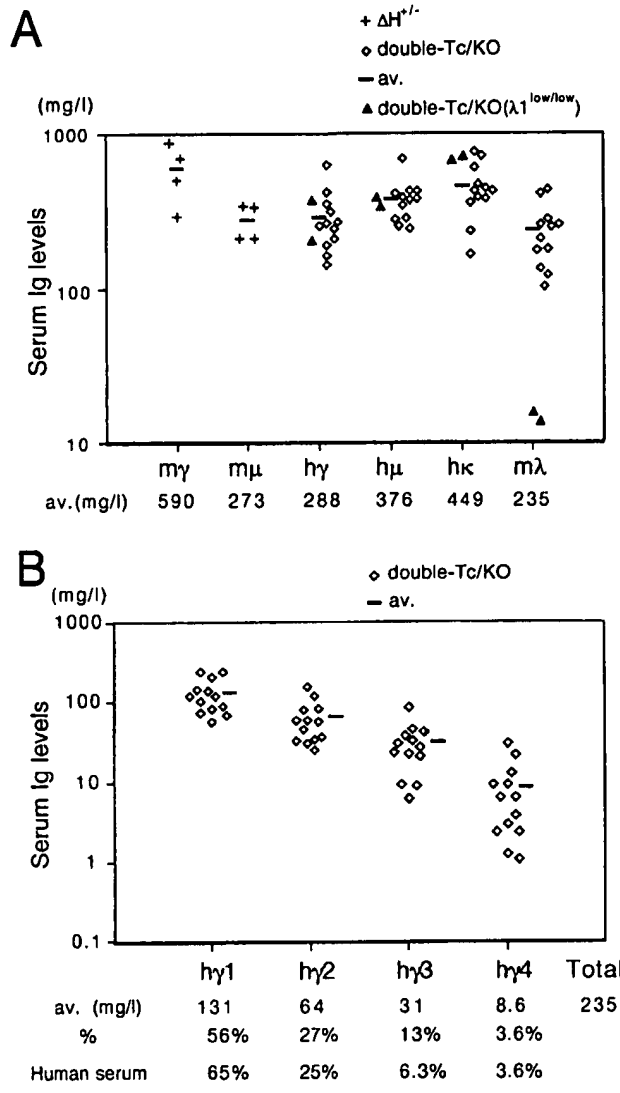

Fig. 4. Human Ig expression in the sera of nonimmunized double-Tc/KO mice. (*A*) Hγ, hμ, hκ, and mλ in serum samples prepared from 8- to 13-week-old double-Tc/KO (n = 12, $\lambda1^{low/+}$ or $\lambda1^{+/+}$) and double-Tc/KO($\lambda1^{low/low}$) (n = 2) individuals. mμ and mγ from 8- to 13-week-old $\Delta H^{+/-}$ individuals (n = 4). The average level of each Ig (av.) in double-Tc/KO or $\Delta H^{+/-}$ individuals is also given below the graph. mγ and mμ levels in double-Tc/KO mice are <15 mg/liter and <1 mg/liter, respectively. (*B*) Four hγ subclasses from the double-Tc/KO individuals (n = 13). The average level of each hγ subclass (av.) is also given below the graph. The percentage of the average concentration for each hγ subclass to the total of them in double-Tc/KO mice and control human serum (Zymed) are also shown.

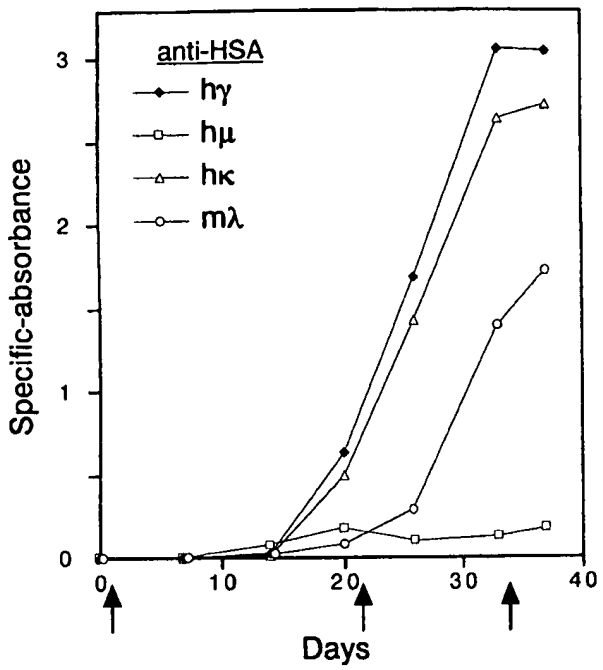

Fig. 5. Time course of antigen-specific human antibody responses in double-Tc/KO mouse. Shown are HSA-reactive hγ, hμ, hκ, and mλ in the serum of a representative double-Tc/KO animal (10 weeks old, male) immunized on days 0, 21, and 34 (indicated with arrows).

double-Tc/KO animals, respectively. Considering that the normal levels of Igα chain are similar to those of Igμ chain in the sera of mice and humans, observed levels of Igα chain in the double-Tc/KO mice are very low. Although the reason for this phenomenon is elusive at present, it should be noted that such a selective defect in IgA production (IgA deficiency) is the most common form of primary immunodeficiency in human, in which an impaired class-switching to IgA is supposed to be involved in the pathogenesis (16). The average level of hκ was higher than that of the mouse λ light chain (mλ), which indicates that the *Igκ* locus of the hCF(2-W23) can compete well with the intact mouse *Igλ* locus despite the incomplete stability of the hCF in somatic cells (Fig. 4*A*). Furthermore, the hκ to mλ ratio was greatly improved by introducing a $\lambda1^{low}$ allele (17) into the double-Tc/KO strain [Fig. 4A, double-Tc/KO($\lambda1^{low/low}$)].

Production of HSA-specific hu-mAbs. The double-Tc/KO mice were challenged with HSA to examine whether the reconstituted repertoire of human Igs is sufficient for obtaining antigen-specific hu-mAbs. After immunization, HSA-specific hγ, hμ, and hκ were readily detected in the sera (Fig. 5). Furthermore, a second immunization resulted in greater hγ and hκ responses. Splenic hybridomas were prepared from one of the HSA-immunized double-Tc/KO animal, and the resulting hybridoma supernatants were screened for the production of HSA-specific human Igs. Analysis of 3,300 wells with hybridomas revealed 11 $h\mu^+$ wells and 39 $h\gamma^+$ wells. Of 39 $h\gamma^+$ wells, 14 were $h\kappa^+$, and the others were $m\lambda^+$. There was no well positive for both Ig light chains, indicating the production of HSA-specific fully human IgG/κ antibodies in these 14 $h\kappa^+$ wells. Further ELISAs of them showed that 7, 2, and 5 wells were $h\gamma1^+$, $h\gamma2^+$, and $h\gamma4^+$, respectively. Representative human IgG/κ hybridomas were subcloned by limiting dilution, and their supernatants were subjected to the affinity measurement. The affinity constant (Ka), measured by using surface plasmon resonance in BIAcore, ranged from 1.1×10^{10} to 6.6×10^{10} M^{-1}.

These results strongly indicate that the double-Tc/KO mice can be used to obtain antigen-specific hu-mAbs with various isotypes exhibiting desired effector functions. Successful expression of all four hγ subclasses represents an advantage of using hCF vectors to bypass cloning steps because some sequences within the constant region of human *IgH* locus was found to be unclonable by conventional cloning systems (6). V gene complexity is supposed to be essential for restoration of normal humoral immune response (5), which is important for the production of high affinity hu-mAbs against variety of antigens. Therefore, high affinities of the resultant hu-mAbs suggest that the authentic repertoire of fully human Igs was reconstituted in the double-Tc/KO mice. Although more

detailed structural analysis of hCFs may be required to determine whether human *Ig* loci contained in the double-Tc/KO mice are completely intact, the data presented here and in our previous report (7) suggest that they include almost all, if not all, of the sequence for the human *Ig* loci. In addition, hybridomas producing human IgG/κ antibodies against human proteins other than HSA, human tumor necrosis factor α (TNFα), and granulocyte colony stimulating factor (G-CSF) have been obtained.

Instability of the hCF(2-W23) could be a impediment to optimal hκ expression and production of hκ^+ hybridomas. Although we successfully obtained anti-HSA hκ^+ hybridomas, the following observations indicate that the loss of the hCF(2-W23) was actually compensated by the mλ expression. (*i*) The proportion of mλ to total Ig light chain concentration in the sera of double-Tc/KO mice [average: 34% (Fig. 4)] was higher than those in wild-type and YAC-transgenic mice (<10%) (5). (*ii*) Delayed but significant response of mλ against HSA-immunization was observed (Fig. 5). (*iii*) Two-thirds of anti-HSA IgG hybridomas obtained were mλ^+. (*iv*) A majority (83%) of IgG/mλ hybridomas was found to have lost the hCF(2-W23). Therefore, the use of more stable hCF with the *Ig*κ locus should be desirable for improving the hκ expression and the production of antigen-specific fully human monoclonals. Site-directed translocation of hCF containing the *Ig*κ locus to a mouse chromosome in ES cells (18, 19) may be one of the solutions for this issue.

There has been no report describing transmittable foreign chromosomes in mice since we demonstrated the transmission of hCF(2-W23) through the germ line of male and female chimeras (7). Now, the hCF(SC20) has been shown to be a second transmittable hCF. The result that the hCF(SC20) could be transmitted through the germ line, whereas the larger hCFs derived from hChr.14 failed (7), implies that the use of small hCFs ($\approx$20 Mb) may increase the probability of successful germ-line transmission. The monochromosomal hybrid library we generated previously (7) can be screened to obtain such small hCFs containing the desired loci. Hence, the procedure presented here should give us an application to humanize other large loci or gene clusters (e.g., T-cell receptors, major histocompatibility antigens, P450 gene clusters) and, ultimately, specific chromosomal regions sintenic between human and mouse (20) in combination with procedures to generate large chromosomal deletions in the mouse (21, 22). Furthermore, an increasing amount of sequence information provided by the Human Genome Project should facilitate not only engineering the hCFs with desired chromosomal regions (15, 23) but also elucidating the structural basis required for stable maintenance and germ-line transmission of the hCFs in mice.

Although the ES cells with germ-line differentiating potency are currently available only in the mouse, the chromosome transgenesis in large farm animals may be feasible using the cloning technology (24, 25). For example, cows or sheep producing human Igs may be generated by nuclear transfer from the microcell-hybrid fibroblast cells retaining the hCFs with human *Ig* loci, from which pathogen-specific human γ-globulins would be obtained for the treatment of infectious diseases. Because the use of hCFs allows for the expression of all four human IgG subclasses, our procedure may be most suitable for this purpose.

Our study has demonstrated the utility of the Tc technology as a complementary approach to conventional transgenic techniques using the cloned DNA fragments. Such a "top-down" approach (15) should be a promising way for large-scale genome manipulations of mice or other animals to generate genetically modified animals useful for laboratory and industrial uses.

We thank K. Hanaoka, M. Hayasaka, and Y. Kuroiwa for technical advice and valuable discussions; H. Masumoto for providing PCR primers to amplify mouse major and minor satellite sequences; and R. Akasu and M. Hirayama for technical assistance.

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